

From the Department of Laboratory Medicine  
Karolinska Institutet, Stockholm, Sweden

# **FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF THE HUMAN MITOCHONDRIAL HELICASE.**

Jenny Korhonen



**Karolinska  
Institutet**

Stockholm 2007

Published and printed by Karolinska University Press  
Box 200, SE-171 77 Stockholm, Sweden  
© Jenny Korhonen, 2007  
ISBN 978-91-7357-101-2

## ABSTRACT

Mitochondria are the energy producing organelles of the eukaryotic cell. The human mitochondrial DNA (mtDNA) is a double stranded circular molecule of about 16 kb, usually present at 1000-10 000 copies per cell. The genome encodes 13 polypeptides involved in respiration, two ribosomal RNA's and a set of 22 transfer RNA's. Nuclear genes encode all the factors required for the transcription and replication of the mtDNA genome. Mutations in mtDNA replication factors are associated with human diseases affecting mitochondrial genome stability and maintenance. The general aim of this thesis has been to investigate the molecular mechanisms of human mtDNA replication with a focus on the recently identified TWINKLE protein, a mitochondrial DNA helicase with 5' to 3' directionality and distinct substrate requirements.

TWINKLE displays sequence similarity to the bacteriophage T7 gene 4 protein, which contains the DNA helicase and primase activities needed at the bacteriophage DNA replication fork. TWINKLE alone is unable to unwind longer stretches of double-stranded DNA (dsDNA), but forms together with the mitochondrial DNA polymerase  $\gamma$  (POL $\gamma$ ) a processive replication machinery, which can use dsDNA as template to synthesize single-stranded DNA (ssDNA) molecules of about 2 kb. Addition of the mitochondrial ssDNA-binding protein stimulates the reaction further, generating DNA products of about 16 kb, the size of the mammalian mtDNA molecule.

Mutations in both POL $\gamma$  and TWINKLE can cause autosomal dominant progressive external ophthalmoplegia (adPEO) with multiple deletions of mitochondrial DNA. Detailed analysis of seven different adPEO-causing mutations in the linker region of TWINKLE reveals different molecular phenotypes, with distinct consequences for multimerization, ATPase activity, DNA helicase activity, and ability to support mtDNA synthesis *in vitro*. A structural model of TWINKLE that is based on the extensive primary sequence similarities with the T7 gene 4 protein is used to explain these distinct molecular phenotypes. In this model, the TWINKLE linker region consists of two helical regions connected by a short turn. The N-terminal helical region makes intra-molecular contacts within the primase domain, whereas the second helical region interacts with the helicase domain of the neighboring monomer. The model suggests that mutations carried out in the first helical region may rather result in miss-folding or destabilization of the monomer itself, whereas mutations in the second helical region of the linker will weaken TWINKLE dimerization, which in

turn affects hexamerization. Biochemical analyses of adPEO causing amino acid changes in the linker region support this interpretation.

Four different adPEO-causing mutations in the N-terminal region of TWINKLE do not affect protein hexamerization, but display severely reduced ATPase activities. The decrease in ATPase activity can be partially overcome by the addition of ssDNA. The structural model of TWINKLE contains a conserved, positively charged surface region that has been implied in binding to ssDNA. All four adPEO-causing amino acid substitutions are located to this region and three of the mutants also display reduced binding to ssDNA *in vitro*.

**Keywords:** Mitochondria, mtDNA, replication, replisome, TWINKLE, mtSSB, POL $\gamma$ , adPEO.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

**I. TWINKLE has 5' -> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein.**

Korhonen JA, Gaspari. M, and Falkenberg M.

*J. Biol. Chem.*, 2003 Dec 5; 278 (49):48627-32.

**II. Reconstitution of a minimal mtDNA replisome *in vitro*.**

Korhonen JA, Pham XH, Pellegrini M, Falkenberg M.

*EMBO J*, 2004 Jun 16; 23 (12):2423-9.

**III. Structure-function defects of the TWINKLE linker region in progressive external ophthalmoplegia**

Korhonen JA, Pande V, Holmlund T, Farge G, Pham XH, Nilsson L, and Falkenberg M

Submitted to *EMBO J*, Jan 2007.

**IV. Structure-function defects of the TWINKLE amino-terminal region in progressive external ophthalmoplegia**

Holmlund T, Farge G, Pande V, Korhonen JA, Pham XH, Nilsson L, and Falkenberg M

Manuscript will be submitted in Jan 2007

# TABLE OF CONTENTS

<b>ABSTRACT.....</b>	<b>3</b>
<b>LIST OF PUBLICATIONS.....</b>	<b>5</b>
<b>LIST OF ABBREVIATIONS.....</b>	<b>8</b>
<b>INTRODUCTION.....</b>	<b>9</b>
Mitochondria.....	9
Evolution of mitochondria.....	10
Mitochondrial energy production.....	10
The mitochondrial genome.....	12
Mammalian mtDNA structure.....	12
Gene content and organization.....	13
Transcription of mtDNA.....	14
Heavy and light-strand promoters.....	15
Factors required for mtDNA transcription.....	16
Introduction to the field of DNA replication.....	17
Replisome structure and composition.....	17
The T7 replisome.....	18
Mechanisms of hexameric DNA helicases.....	19
The T7 gene 4 protein.....	20
Replication of mtDNA.....	21
Strand-displacement model of replication (Clayton model).....	21
Strand-coupled model of replication (Holt model).....	22
RITOLS –a new mode of replication.....	23
Critical elements involved in mtDNA replication.....	24
Factors required for mtDNA replication.....	24
DNA polymerase $\gamma$ holoenzyme .....	24
Mitochondrial single stranded DNA-binding protein.....	25
TWINKLE-the human mitochondrial DNA helicase.....	26
Other enzymatic activities required for mtDNA replication.....	26
Inheritance and segregation of mtDNA.....	28
Mitochondrial disorders.....	28
Mitochondrial DNA mutations.....	29

Nuclear gene mutations affecting mtDNA stability.....	29
Autosomal dominant progressive external ophthalmoplegia.....	30
<b>AIMS OF THIS THESIS.....</b>	<b>31</b>
<b>RESULTS AND DISCUSSION.....</b>	<b>32</b>
Biochemical characterization of human TWINKLE <i>in vitro</i> (Paper I).....	32
Reconstitution of the mammalian mtDNA replisome <i>in vitro</i> (Paper II).....	32
Analysis of adPEO-causing linker region mutants of TWINKLE (Paper III).....	33
Analysis of N-terminal adPEO mutants of TWINKLE (Paper IV).....	34
<b>CONCLUDING REMARKS.....</b>	<b>37</b>
<b>PERSPECTIVES.....</b>	<b>38</b>
<b>ACKNOWLEDGEMENTS.....</b>	<b>39</b>
<b>REFERENCES.....</b>	<b>41</b>

## LIST OF ABBREVIATIONS

aa	Amino acids
adPEO	autosomal dominant Progressive External Ophthalmoplegia
ANT	Adenine nucleotide translocator
ATF	Atomic force microscopy
ATP	Adenosine triphosphate
bp	Base pair
COX	Cytochrome C oxidase
CSB	Conserved sequence block
D-loop	Displacement loop
dsDNA	Double-stranded DNA
GFP	Green fluorescent protein
HSP	Heavy-strand promoter
kDa	Kilo Dalton
LSP	Light-strand promoter
mtDNA	Mitochondrial DNA
mtSSB	Mitochondrial single stranded DNA binding protein
nt	nucleotide
O <sub>H</sub>	Origin of heavy-strand
O <sub>L</sub>	Origin of light strand
PGC-1 $\alpha$	PPAR $\gamma$ coactivator-1 $\alpha$
POL $\gamma$	Mitochondrial DNA polymerase (holoenzyme)
POLRMT	Mitochondrial RNA polymerase
RC	Respiratory chain
RI	Replication intermediate
ssDNA	Single-stranded DNA
TFAM	Mitochondrial transcription factor A
tRNA	Transfer RNA



# INTRODUCTION

## Mitochondria

All human cells require energy to function. This energy is generated by the powerhouse of eukaryotic cells, the mitochondrion. Mitochondria are organelles of prokaryotic origin that are present in most eukaryotic cells. They take part in a dynamic tubular network, in which they constantly divide and fuse. Each mitochondrion is bounded by two membranes, separated by an intermembrane space. The inner membrane forms a series of invaginations called tubular cristae that extensively increase the surface area of the mitochondrial inner membrane. The inner membrane is especially impermeable to ions thereby maintaining the proton gradient that drives oxidative phosphorylation. The interior space of mitochondria, the so called mitochondrial matrix, is the site where several copies (2-10) of the mitochondrial DNA (mtDNA) genome can be found. In addition, the matrix also contains mitochondrial ribosomes, transfer RNAs (tRNAs) and a highly concentrated mixture of hundreds of enzymes required for mitochondrial function. Mitochondria are involved in a variety of processes in the cell such as: lipid metabolism, Krebs cycle, apoptosis, formation of reactive oxygen species and calcium buffering. However, the dominant role for the mitochondria is the production of ATP as reflected by the large number of proteins embedded within the inner membrane responsible for this task. This is done by oxidizing the major products of glycolysis: pyruvate and NADH that are produced in the cytoplasm. This process of cellular respiration is dependent on the presence of oxygen. The production of ATP from glucose has an approximately 15 fold higher yield during aerobic respiration compared to anaerobic respiration. Due to the many fundamental functions in the cell, malfunction of mitochondria give rise to many harmful effects on cells and organisms. The first case of a mitochondrial disease, named Luft disease, was described over 40 years ago in a patient, with severe non-thyroidal hypermetabolism (Luft et al., 1962). Twenty years later, the first pathogenic point mutation (Wallace et al., 1988) and large-scale deletions of mtDNA (Holt et al., 1988) were identified. Since then the number of human diseases linked to mutations in the mitochondrial DNA or proteins involved in mtDNA maintenance has increased continuously. Furthermore, mitochondrial dysfunction may also be involved in neurodegenerative diseases such as Parkinson's and Alzheimer's disease, diabetes mellitus and even in the natural ongoing aging process (Trifunovic et al., 2004).

## Evolution of Mitochondria

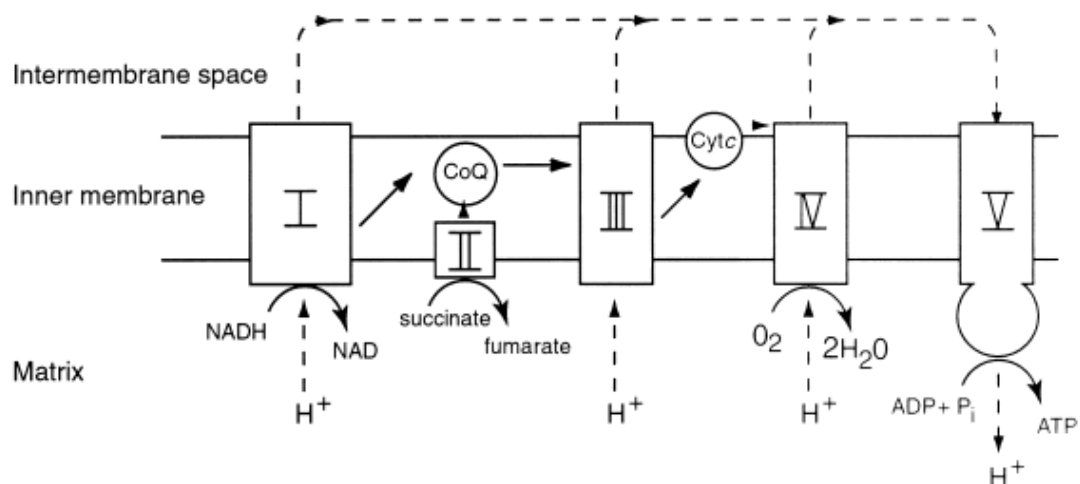
According to the endosymbiotic theory, now generally accepted by biologists, mitochondria originated from a single ancient invasion of an Archea-type host by a  $\alpha$ -proteobacterium-like ancestor over 2 billion years ago. The two organisms developed a symbiotic relationship over time, which was probably driven by metabolic requirements (Gray et al., 1999). Most likely this  $\alpha$ -proteobacterium was related to what we today know as *Rickettsia prowazekii*. *Rickettsia prowazekii* is an obligate intracellular parasite causing epidemic typhus in human. Its genome has a size of 1.1Mbp DNA and encodes for 834 proteins. The genome encodes a complete set of proteins for aerobic respiration, ATP production and ATP transport (Andersson et al., 1998). Most of the genes found in mtDNA today represent genetic information retained from the original endosymbiont.

However, it cannot be excluded that some genetic information was acquired later in time after the symbiosis had been established. A witness of this type of event is the brown alga *Pylaiella littoralis*. This alga contains an entire T7-phage-type RNA polymerase gene integrated in the mitochondrial genome (Rousvoal et al., 1998). It is still an open question when the phage-encoded RNA polymerase was acquired. It could have either pre-existed in the ancestral  $\alpha$ -proteobacterium or been recruited later on in the evolution of mitochondria. In animal, fungi and plants the phage-type RNA polymerase has been transferred from the mitochondria into the nucleus.

## Mitochondrial energy production

The major energy currency of the cell, ATP, is produced by mitochondria through a process known as oxidative phosphorylation taking place in the mitochondria inner membrane. The major energy nutrient in our diet is glucose (six-carbon sugar). One molecule of glucose is initially degraded in a process called glycolysis, which occurs in the cytoplasm yielding 2 pyruvate molecules, 2 NADH and 2 ATP molecules whereas the remaining 30 ATP molecules are generated through oxidative phosphorylation. Oxidation of food materials, such as sugars, fats and amino acids is funneled into the formation of two energy-rich electron donor end products within mitochondria, NADH and FADH<sub>2</sub>. Electrons from these donors are passed through an electron transport chain to the final electron acceptor, oxygen which is reduced to water. The enzymes that catalyze these reactions constitute for the respiratory chain

(RC) and consist of five multimeric protein complexes, named complex I-V (Figure 1). The RC also requires two small electron carriers, coenzyme Q (CoQ) and Cytochrome c (Cyt *c*). Electrons are fed into complex I (NADH dehydrogenase) or II (succinate dehydrogenase) and transferred to complex III and IV. During the passage of electrons a proton gradient across the inner membrane is built up by proton translocation at complex I, III and IV. This generates a proton gradient and a transmembrane electrical potential across the membrane, a so called proton-motive force is thus generated (Mitchell, 1961; Racker, 1980). Complex V or ATP synthase harvest the energy by using the proton gradient that was built up to generate ATP from ADP and phosphate by the influx of protons back into the mitochondrial matrix. The RC consists of ~90 different subunits with the majority being nuclear encoded and the minority, 13 subunits being encoded by the mitochondrial genome. The mitochondrial genome in humans encodes for subunits in four out of five complexes. Complex II is entirely nuclear-encoded. Recent studies suggest that the respiratory complexes are organized in supercomplexes, referred to as “respirasomes” and these have been found in mitochondria of yeast, plants and mammals (Krause et al., 2004; Schafer et al., 2006; Schagger and Pfeiffer, 2000).



**Figure 1. Schematic illustration of the respiratory chain.**

The respiratory chain consists of five different enzyme complexes (Complexes I-V), and two electron carriers, coenzyme Q (CoQ) and cytochrome c (cyt *c*). Electrons are fed into either complex I or II. Protons (H<sup>+</sup>) are pumped out of the matrix into the intermembrane space and are reentered via complex V, which uses the energy of the proton gradient to generate ATP. Complex II does not translocate protons and is entirely nucleus-encoded (Larsson and Clayton, 1995).

## **The mitochondrial genome**

In addition to the nuclear genetic material, most eukaryotic cells also contain a mitochondrial genome. Mitochondrial DNA was first discovered in the 1960s (Nass and Nass, 1963) and about twenty years later the human mtDNA was completely sequenced (Anderson et al., 1981). Later studies describe the complete sequence of mtDNA from many animal species (Wolstenholme, 1992). Cells are polyploid with respect to mtDNA and most mammalian cells contain between hundreds to thousands copies of its genome. In a given cell of an individual, all mtDNA copies are presumably identical, a condition called homoplasmy. Mutations can however arise and be maintained or amplified to varying levels thereby coexisting with wild-type mtDNA, a condition called heteroplasmy. Since there is a random distribution of mtDNA to daughter cells during cell division, different levels of heteroplasmy and even homoplasmy can arise in different cell lineages (Lightowlers et al., 1997).

### ***Mammalian mtDNA structure***

A detailed picture of the molecular and structural organization of mammalian mtDNA does not exist to date but it was early demonstrated that mtDNA in yeast, distinct from the nuclear genome, lacks histones (Caron et al., 1979). The normal mtDNA state is thought to be super-coiled associated with a highly reduced number of proteins. It has been suggested that mtDNA is fully coated by the mitochondrial transcription factor A (TFAM), having a histone-like function (Alam et al., 2003; Fisher et al., 1992; Takamatsu et al., 2002). However, mtDNA is not spread out homogeneously in the mitochondrial matrix. The mtDNA is organized in protein-DNA structures called nucleoids, which in turn are associated with the mitochondrial inner membrane (Albring et al., 1977; Iborra et al., 2004). The functional basis for nucleoids and their association with the membrane is not completely clear, but they may have roles in inheritance, segregation, replication, transcription and perhaps even recombination.

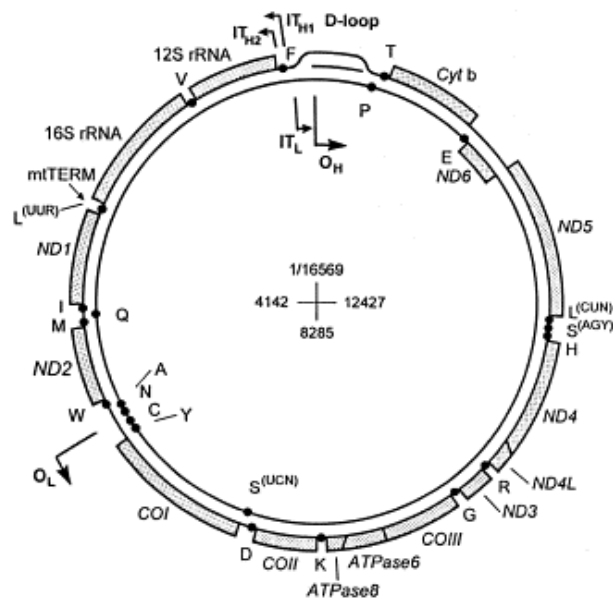
Nucleoids composition and function have mainly been studied in yeast. Yeast nucleoids contain several (3-4) mtDNA molecules and more than 20 different polypeptides are found to be associated with the mitochondrial membranes (Chen et al., 2005; Hobbs et al., 2001; Kaufman et al., 2000). One of the core components found in yeast nucleoids was the *abf2* protein, an orthologue of the human mitochondrial transcription factor A (TFAM). Another yeast component was Rim1p, the yeast single-stranded DNA binding protein (Kaufman et al., 2000). The existence of nucleoids in mammalian cells was also

recently demonstrated. The putative human mtDNA helicase, TWINKLE was the first protein found associated with human nucleoids *in vivo* (Spelbrink et al., 2001). A partial co-purification of enriched nucleoids from cultured human cells revealed the presence of replication factors, such as the mitochondrial TWINKLE, mtSSB, and TFAM associated with the nucleoid structure (Garrido et al., 2003). A recent fluorescence study in human cells revealed the distribution of hundreds of punctuated nucleoids (~300-800) regularly scattered within the mitochondrial compartment (Malka et al., 2006). In another recent study by Bogenhagen and co-workers, a purification scheme was reported for the isolation of mtDNA nucleoids from cultured human HeLa cells and associated proteins were identified. They found that mitochondrial nucleoids are quite heterogeneous in nature, comprising two major subsets of complexes, which differed in their sedimentation velocity. Overall, they found a set of ~20 proteins involved in mtDNA maintenance, such as TFAM, mitochondrial RNA polymerase, mtSSB, and a novel DEAH helicase, DHX30, (Wang and Bogenhagen, 2006).

### *Gene content and organization*

Human mtDNA is a double-stranded, closed-circular molecule of about 16.6kb in size (Figure 2). The two strands of the DNA duplex are called heavy (H) strand and light (L) strand, based on differences in their G + T content allowing them to be separated by density in denaturing gradients (Taanman, 1999). The human mitochondrial DNA contains a total of 37 genes coding for 2 ribosomal RNAs (rRNA), 22 transfer RNAs (tRNA) and 13 polypeptides. All 13 peptide products are essential subunits of respiratory complexes I, III, IV, and V. The 22 tRNAs are sufficient for the translation process of mtDNA, due to a simplified codon-anticodon pairing system occurring within the matrix. The genome displays a very economical organisation, it lacks introns, has some protein-encoding overlapping genes and incomplete termination codons (Anderson et al., 1981). There is one major non-coding region in human mtDNA, called the displacement loop (D-loop). The D-loop which is about 1kb in humans is a major control region for mtDNA expression containing both the origin of heavy strand replication ( $O_H$ ) as well as the promoters for transcription of both strands (Figure 3). The D-loop is a triple stranded structure containing a nascent arrested H-strand DNA segment of 500-700 nt (called the 7S DNA) which remains annealed to the L-strand while the parental H-strand is displaced as a loop. A second shorter non-coding region, the origin of light strand ( $O_L$ ) is located about two-thirds downstream of  $O_H$ , positioned

within a tRNA cluster (Fernandez-Silva et al., 2003; Hixson et al., 1986; Taanman, 1999)



**Figure 2. The gene content and organization of the human mitochondrial DNA.**

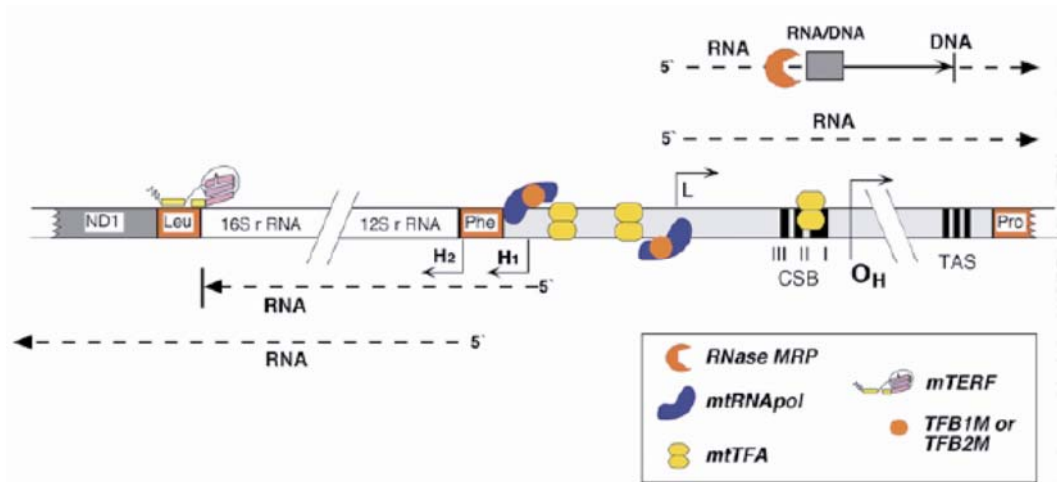
The outer circle represents the heavy-strand (H-strand) and the inner circle represents the light-strand (L-strand). The initiation of transcription sites ( $IT_L$ ,  $IT_{H1}$ ,  $IT_{H2}$ ) and the direction of RNA synthesis are denoted by short bent arrows. The D-loop is shown as a three-stranded structure. The origins of H-strand ( $O_H$ ) and L-strand ( $O_L$ ) replication and the direction of DNA synthesis are indicated by long bent arrows. The 22 tRNA genes are depicted by dots and the genes are depicted by shaded boxes (Taanman, 1999).

Mitochondria are however not self-supporting organelles but rely on a vast number of nuclear encoded factors for proper function. Nuclear genes encode many critical proteins such as; the remaining RC subunits, proteins required for mtDNA transcription, replication and translation as well as mitochondrial transport. All nuclear proteins destined for the mitochondria are synthesized in the cytoplasm and then directed and transported into the mitochondria. Most of the nucleus encoded mitochondrial proteins are directed to the mitochondria through an N-terminal cleavable pre-sequence (Shadel and Clayton, 1997).

## Transcription of mtDNA

In humans, transcription is initiated from 2 different promoters in the D-loop., one in the L-strand (light-strand promoter, LSP) and one (with two initiation sites) from the

H-strand (heavy-strand promoter, HSP<sub>1</sub> and HSP<sub>2</sub>) (Bogenhagen et al., 1984; Montoya et al., 1982). The HSP and LSP promoters are located within 150 bp of one another, but despite their proximity both are functionally independent. Large polycistronic transcripts are produced from each strand and then further processed to generate mature tRNAs, rRNAs, and mRNAs (Shadel and Clayton, 1997).



**Figure 3. The D-loop controls replication and transcription of mtDNA.**

The main elements and proteins involved in initiation of transcription and replication are represented in the figure. The non-coding D-loop is shown in light grey. The H<sub>1</sub> initiation point directs the transcription of the rRNA region and is linked to a termination event by mTERF. The H<sub>2</sub> direct transcription of the whole H-strand and is 20 times less active than H<sub>1</sub>. L is the light-strand initiation point which gives rise to a polycistronic transcript. mtTFA (TFAM) binding to enhancer elements and the CSB region is shown. TAS, represents the site where nascent H-strand DNA molecules arrest, giving rise to the D-loop structure (Fernandez-Silva et al., 2003).

### *Heavy and light-strand promoters*

The heavy strand encodes most information of the human mtDNA genome. The HSP<sub>1</sub> initiation site is located 19 nt upstream of the tRNA<sup>Phe</sup> gene and ends at the 16S rRNA 3'-end. This transcription initiation site operates about 20 times more frequently and generates two rRNAs (12S and 16S), and two tRNAs (tRNA<sup>Phe</sup> and tRNA<sup>Val</sup>). The short transcript, initiated from HSP<sub>1</sub> is linked to a termination event located immediately after the 16S rRNA gene, and mediated by specific binding to a tridecamer sequence of a mitochondrial termination factor (mTERF) (Montoya et al., 1983). A recent study has reconstituted mTERF termination activity *in vitro*,

demonstrating that the termination activity has a distinct polarity. Thus, complete transcription termination was observed when the mTERF-binding site was oriented in a forward position relative to the heavy strand promoter but only partial transcription termination was observed when the binding site was in the reverse position (Asin-Cayuela et al., 2005). The second initiation site, HSP<sub>2</sub> is located close to the 12S rRNA 5'-end and initiates transcription of a long polycistronic transcript containing 2 rRNA, 13 tRNAs, and 12 mRNAs (Fernandez-Silva et al., 2003). The light strand promoter (LSP) positioned some 150bp away from H1 initiates a single polycistronic transcript, encoding 8 tRNAs, and one mRNA, corresponding to the ND6 subunit (Montoya et al., 1982). The specific locations for termination of the LSP and the full-length HSP<sub>2</sub> transcripts have not been mapped to date.

### *Factors required for mtDNA transcription*

The nuclear encoded factors responsible for mtDNA transcription in human include the mitochondrial RNA polymerase (POLRMT), a transcription and maintenance factor (TFAM), transcription specificity factors (TFB1M and TFB2M) and a transcription termination factor (mTERF). The two promoters HSP and LSP share an important upstream enhancer that acts as a recognition site for the mitochondrial transcription factor A (TFAM). TFAM, which belongs to the high mobility group box protein family (HMG), can stimulate transcription via its bending and unwinding properties (Fisher et al., 1992; Topper and Clayton, 1989). The importance of TFAM in mtDNA replication and transcription became apparent in studies of homozygous TFAM knockout mice, which display embryonic lethality and depletion of mtDNA (Larsson et al., 1998)

Human POLRMT, a 120 kDa protein, displays a high sequence similarity to both the T3 and T7 bacteriophage polymerases (Masters et al., 1987). With the discovery of the human transcription factors B1 and B2, a successful reconstitution of the basal human transcription machinery was established in a pure *in vitro* system. POLRMT forms a soluble hetero-dimeric complex with either TFB1M or TFB2M. Each complex could independently together with TFAM support promoter-specific initiation of transcription. Moreover, TFB2M was found to be at least 10 times more active than TFB1M in promoting transcription initiation (Falkenberg et al., 2002). Human TFB1M and TFB2M were shown to be closely related to a large class of rRNA



methyltransferases and in accordance with this group of enzymes, h-TFB1M binds the methyl-group donor S-adenocylmethionine (SAM) (McCulloch et al., 2002).

## **Introduction to the field of DNA replication**

Much of what we know today about the molecular mechanisms of DNA replication has been derived from studies of *Escherichia coli* (*E.coli*) and its bacteriophages T7 and T4. All three systems use the same fundamental components for DNA synthesis at the moving replication fork, although the number, nature and the lack of extensive sequence homology between the proteins can vary. Some of the main components are: a DNA polymerase, a polymerase accessory factor, a single-stranded DNA binding protein, and a primosome (DNA helicase and DNA primase activities) (Benkovic et al., 2001). Before mtDNA replication will be discussed in detail a brief introduction to the DNA replication field is given.

## **Replisome structure and composition**

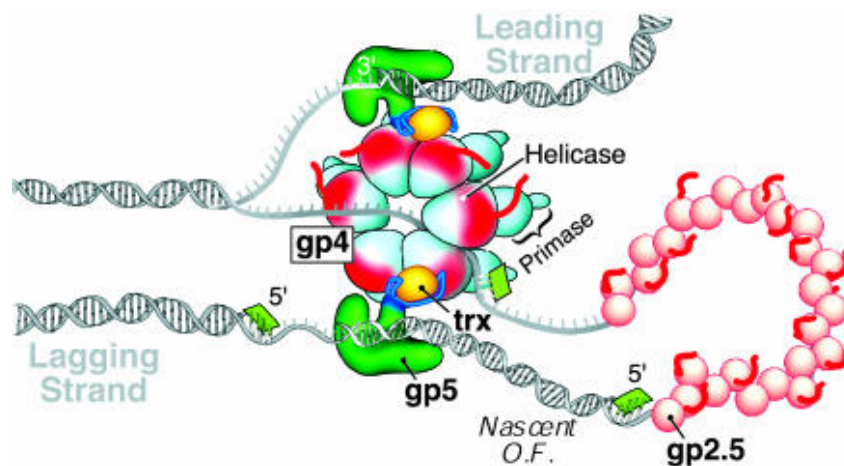
During replication each of the two DNA strands serves as a template for the formation of a new daughter DNA strand. A multi-protein complex, often referred to the replisome, acts together at the DNA replication fork to perform smooth duplication of the genome. The anti-parallel nature of DNA presents a problem for the moving DNA replication fork since DNA polymerases can only synthesize DNA in the 5' to 3' direction. The replisome must accommodate two distinct modes of operation. On the leading DNA strand, the DNA polymerase synthesizes the DNA continuously in the same direction as a helicase. This is not possible on the lagging strand and the DNA polymerase and helicase move in opposite directions. The DNA is instead synthesized in form of multiple short DNA fragments referred to as Okazaki fragments. Each fragment is synthesized in the 5' to 3' direction but the overall movement of the growth of the lagging strand is 3' to 5' relative to the fork.

Furthermore, DNA polymerase cannot initiate DNA synthesis de novo; they need a RNA primer. A primase is therefore constantly needed at the DNA replication fork to initiate DNA synthesis of both the leading strand and every Okazaki fragment on the lagging strand. In order to coordinate the multiple reactions taking place at the replication fork, it is necessary to loop the lagging strand back through the replisome. Otherwise discontinuous synthesis at the lagging strand would be hundreds of

nucleotides distal to leading strand events. Such a “trombone” model was first proposed in the mid 1970 in the bacteriophage T4 phage system (Morris et al., 1975) and subsequently extended to *E-coli* (Wu et al., 1992) and the phage T7 system (Park et al., 1998).

### *The T7 replisome*

Due to the high resemblance between the human mitochondrial replication system and the T7 bacteriophage, a lot can be learnt by studying the T7 phage replisome. To date at least three replication proteins in human mitochondria are related to that of the bacteriophage T7, the mitochondrial helicase TWINKLE, POL $\gamma$  and POLRMT (Lecrenier et al., 1997; Spelbrink et al., 2001; Tiranti et al., 1997). The T7 replisome can be reconstituted with only four proteins (Richardson, 1983) (Figure 4). The functional T7 DNA polymerase, called T7 gene 5 protein (gp5) forms a 1:1 complex with its processivity factor, *Escherichia coli* thioredoxin (Tabor et al., 1987). The hexameric T7 63kDa gene 4 protein (gp4) provides both helicase and primase activities. The acidic C terminus of the helicase domain of gp4 physically interacts with gp5 (Notarnicola et al., 1997; Tabor and Richardson, 1981). Finally, a ssDNA-binding protein is required at the replisome, named the T7 gene 2.5 protein (gp2.5) which coats ssDNA and interacts with both gp5 and gp4 (Kim and Richardson, 1994; Kim et al., 1992).



**Figure 4. Model of the T7 replisome.**

The replisome consists of 4 proteins; DNA polymerase (gp5), the processivity factor thioredoxin (trx), the hexameric helicase/primase (gp4), and the ssDNA-binding protein (gp2.5). The ssDNA of the lagging strand is coated by gp2.5. The lagging strand forms a loop that contains nascent Okazaki fragments. The acidic C termini of gp4 and gp2.5 are shown as red tails (Hamdan et al., 2005).

The four proteins are sufficient for replisome mediated leading- and lagging-strand synthesis in a coordinated matter using a mini-circle template. The discontinuous synthesis at the lagging strand, give rise to multiple Okazaki fragments, with an average length of 3000 nt (Debyser et al., 1994; Lee et al., 1998).

## **Mechanisms of hexameric DNA helicases**

The first helicase was identified in the late seventies in *E. coli* (Abdel-Monem and Hoffmann-Berling, 1976). Since then a vast number of DNA and RNA helicases have been identified with diverse functions in a variety of organisms. Helicases are not only important for DNA replication. They are also involved in other processes such as repair, DNA recombination and transcription. Helicases can be viewed as NTPase coupled motors; hence they are known to couple the chemical energy of NTP hydrolysis in order to separate the two complementary strands of double-stranded DNA (dsDNA). Helicases can be distinguished in many ways, one being based on their oligomeric structure. Hexameric helicases form a ring-shaped structure, and all 12 known hexameric helicases to date, except one are homohexamers (Patel and Picha, 2000).

Helicases are known to have a distinct polarity and translocate unidirectional on a single stranded DNA in either the 5'-3' or 3'-5 direction. Many hexameric helicases, such as DnaB from *E.coli* and the gene41 protein (gp41) of bacteriophage T4 require a helicase loader protein to properly bind to ssDNA. An exception is however the T7 phage gene 4 protein (Donmez and Patel, 2006). Hexameric helicases from different sources use individually preferred co-factors to form functional hexamers. For instance, ATP or GTP binding is required for hexamerization of gp41 (Dong et al., 1995), while DnaB hexamerization is  $Mg^{2+}$ -dependent (Bujalowski et al., 1994) (Table 1). Hexameric helicases display a number of activities that are all required for efficient unwinding. Most activities show an interdependence. For instance, NTP binding stimulate hexamer formation, and hexamer formation and NTP binding are necessary for DNA binding. Moreover, DNA binding normally stimulates NTPase activity and unwinding. The energy transduction, in turn, which couples NTPase activity to movement and unwinding, is dependent on all activities mentioned previously (Guo et al., 1999; Patel and Picha, 2000).

**Table 1.** Some of the known hexameric helicases (Patel and Picha, 2000).

Proteins	Molecular weight	Direction of unwinding	Minimal requirement for hexamer formation
Bacteriophage T7 gp4	4A 62,6 4B 55,7	5'-3'	dTTP, dTDP, dTMP-PCP, ATP, dATP
Bacteriophage T4 gp41	53,6	5'-3'	ATP, GTP, ATP $\gamma$ S, GTP $\gamma$ S
B.subtilius phage SPP1	46,7	5'-3'	ATP, Mg <sup>2+</sup>
E.coli DnaB	52,9	5'-3'	Mg <sup>2+</sup>
Human Bloom's syndrome helicase	159	3'-5'	ATP $\gamma$ S, Mg <sup>2+</sup>
Simian virus large T antigen	81,9	3'-5'	ATP, ADP, AMP $\gamma$ NP, ATP $\gamma$ S

### *The T7 gene 4 protein*

Twinkle and the T7 gp4 protein display a high homology and the phage helicase therefore serves as a good model for understanding the role of TWINKLE.

Interestingly, the bacteriophage T7 helicase has been well studied in the past and its structure and biochemical properties have been analyzed in detail. The gene 4 bacteriophage T7 encodes two proteins, gp4a (63kDa) and gp4b (56kDa), translated from separated in frame start sites. The gp4A has both primase and helicase activities and the truncated gp4B has only helicase activity (Bernstein and Richardson, 1989; Dunn and Studier, 1983). The helicase activity resides within the carboxyl- terminal half of the protein and the primase activity in the amino-terminal half. The intervening linker region is believed to be critical for hexamer formation (Frick et al., 1998; Guo et al., 1999; Mendelman and Richardson, 1991).

Electron microscope studies showed that T7 assembles in to a ring-shaped helicase with a ring diameter of 13nm in the presence of its preferred nucleotide cofactor dTTP (Egelman et al., 1995). Later, crystal structures revealed that the nucleotide cofactors bind at the interfaces between the monomers (Sawaya et al., 1999; Singleton et al., 2000). The T7 helicase moves at its maximal speed along ssDNA, but upon encountering dsDNA it slows down remarkably. However, having the DNA polymerase present at the replication fork junction accelerates the unwinding rate of T7 helicase, indicating functional coupling of the two motor proteins (Jeong et al., 2004; Stano et al., 2005).

## Replication of mtDNA

MtDNA is continuously amplified independently from the cell cycle and the nuclear genome (Bogenhagen and Clayton, 1977). Additionally, it has been shown that mtDNA replication does not follow the growth and division of the organelles (Shadel and Clayton, 1997). Mitochondria replicate and divide mainly in response to the energy requirements of the cell. The mechanism by which mtDNA levels are regulated in human cells is not known. Since regulation of mtDNA copy number is closely related to the regulation of mtDNA replication, all nuclear encoded replication factors are obvious potential candidates for maintenance of mtDNA copy number. Moreover, since transcription is coupled to mtDNA replication by providing a RNA primer necessary to initiate DNA synthesis, transcription factors are also potentially candidates for copy number control.

To date, the exact mechanisms by which mammalian mtDNA is replicated is still a controversy and two different models of replication have been suggested for mammalian mtDNA replication: the 'strand-displacement model' (or Clayton model) (Clayton, 1982) and the more recent 'strand-coupled' model (or Holt model) (Holt et al., 2000) (Figure 5). The Holt model was recently modified and was shown to include a second mode of replication, where they observed frequent ribonucleotide incorporation throughout the lagging strand, or RITOLS for short (see below). The strand-displacement model and the strand-coupled model have not yet been reconciled and are not compatible with each other. There is an intense debate about the mechanisms and it is evident from both sides that further experimental work is needed to clarify the situation (Bogenhagen and Clayton, 2003a; Bogenhagen and Clayton, 2003b; Holt and Jacobs, 2003).

### *Strand-displacement model of replication (Clayton model)*

The strand-displacement model is based on a large amount of data from studies of replication intermediates (RI) from the 1970s. These RI were characterized by numerous pulse and pulse-chase assays (Berk and Clayton, 1974; Berk and Clayton, 1976) and also by direct visualization with the electron microscope (Kasamatsu et al., 1971; Robberson and Clayton, 1972). According to this model (also called the asymmetric model for mtDNA replication), DNA synthesis is continuous on both strands but the synthesis is separate in both space and time. DNA replication is initiated from two separate unidirectional origins, one for each strand,  $O_H$  and  $O_L$ . The

DNA synthesis is first initiated at  $O_H$ . Transcription is an integral part of mtDNA replication since the RNA primer required for initiation is produced by transcription from LSP. Interestingly, only a few initiation events at  $O_H$  leads to the synthesis of a full-length genome, whereas as much as 95% of initiation events are terminated at the termination associated sequences (TAS) (Fig. 3). Hence the TAS region serves as a major regulatory domain for mtDNA replication (Madsen et al., 1993).

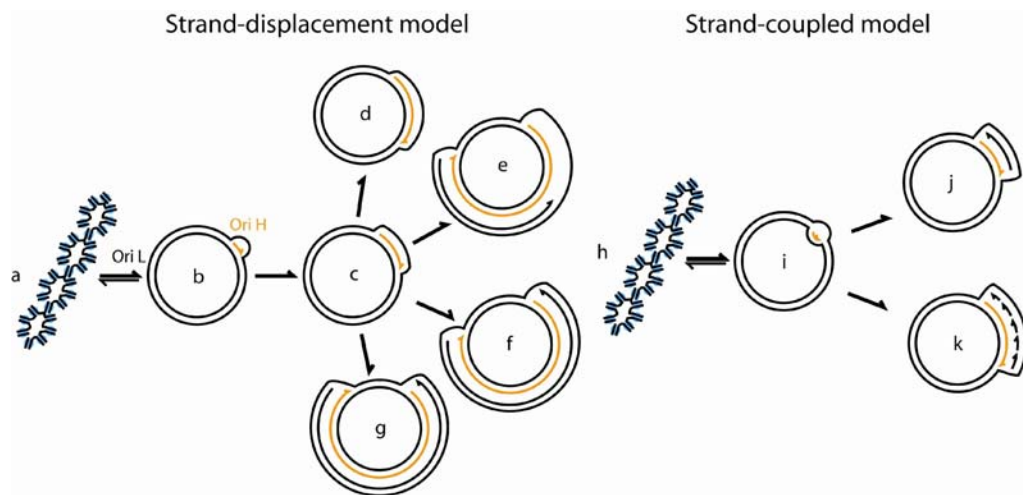
The synthesis of the new H-strand proceeds until a full circle is produced. DNA replication from  $O_L$  is not initiated until the nascent H-strand reaches this origin and  $O_L$  is exposed as a single stranded stem-loop structure. The precise mechanisms for DNA initiation at  $O_L$  are still unclear but it has been postulated that a yet to be identified primase places a primer at the single-stranded  $O_L$ . L-strand DNA synthesis is then initiated in the opposite direction and proceeds in a continuous way until full-length mtDNA is synthesized. The mechanisms by which the two circular duplex DNAs segregate are not clear (Shadel and Clayton, 1997; Wong and Clayton, 1985).

In a recent study from the Clayton laboratory, they tried to validate the strand-displacement model for mammalian mtDNA replication using similar techniques as those used by Holt and colleges. They examined mtDNA RIs from mouse liver using procedures that produced typical y-arc patterns detected by 2D gel techniques and these molecules were further examined using atomic force microscopy (ATF). They concluded from the data obtained that the strand-displacement mode of replication is the dominant or even sole mode of replication. In addition, they found some new data from ATF mapping, which indicated that alternative L-strand initiation sites exist besides the orthodox  $O_L$ . The authors speculate that previous studies (Tapper and Clayton, 1981; Tapper and Clayton, 1982) probably failed to identify other 5'-ends by mapping from cell culture systems, could indicate that these initiation sites are broadly distributed and less abundant than those at the major  $O_L$  site in the cell line used, thereby making them more difficult to detect (Brown et al., 2005).

#### *Strand-coupled model of replication (Holt model)*

A second model, the so called strand-coupled or symmetric model, was first described 2000 and has been modified in several steps since then (Bowmaker et al., 2003; Holt et al., 2000; Yang et al., 2002; Yasukawa et al., 2006; Yasukawa et al., 2005). This model has been mainly based on neutral two-dimensional agarose gel electrophoresis

(2-D AGE) of mitochondrial DNA replication fork movement, which indicates the existence of coordinated leading and lagging-strand DNA synthesis. Double stranded RIs were predominantly found, suggestive of a conventional coupled leading- and lagging-strand synthesis mode of replication. Additionally, some of the double-stranded RIs contained frequent patches of ribonucleotides, suggestive of multiple priming sites on the lagging strand, as would be expected in this model. Coupled replication was at first believed to initiate at or near the  $O_H$  and then proceed in a unidirectional fashion around the mtDNA molecule. Later studies demonstrated that replication starts from multiple sites (*Ori*) across a broad zone and subsequently proceeds bidirectional.  $O_H$  provides fork arrest and replication is then restricted to only one direction.



**Figure 5.** The two different models of replication (Adapted from (Clayton, 2003))

#### *RITOLS –a new mode of replication*

Recently the symmetric model was modified even further to include an additional mode of replication observed in birds and mammals, frequently entailing ribonucleotide incorporation throughout the lagging strand (RITOLS). Initiation of RITOLS replication occurs strictly from the major non-coding region of vertebrate mtDNA and was shown to be unidirectional. Both the strand-asynchronous and RITOLS mode of replication have in common a delayed synthesis of the DNA lagging-strand. However RNA is deposited on the displaced H-strand, thus forming

RITOLS, rather than mtSSB which is suggested in the strand-asynchronous model. The DNA subsequently replaces the RNA, with an unknown mechanism to produce the dsDNA. The authors speculate that the physiological significance of RITOLS could be to stabilize the displaced DNA strand and protect it against damage during replication. Another purpose for RITOLS could involve a regulatory role, where they would act as roadblocks to arriving transcription complexes, thereby arresting transcription until DNA replication is completed (Yasukawa et al., 2006).

### *Critical elements involved in mtDNA replication*

As mentioned above, initiation of DNA replication at  $O_H$  is coupled to the transcription. Mitochondrial replication is therefore not only dependent on the function of the mitochondrial replication machinery but also the mitochondrial transcription machinery. The CSBs refer to conserved sequence blocks (termed CSB I, CSB II and CSB III). They are located in the D-loop downstream of LSP and have been found to be highly conserved among vertebrates, suggesting important roles for these sequences. Stable and persistent RNA-DNA hybrid formation has been observed at the human mitochondrial  $O_H$ , known as an R-loop. As predicted, hybrid formation was dependent on the GC-rich CSBII element. The RNA-DNA hybrids terminated within or downstream of CSBII at locations implicated in initiation of mitochondrial DNA replication (Chang and Clayton, 1985; Pham et al., 2006; Xu and Clayton, 1995). In addition to the CSBs, the TAS also seems to participate in the replication process. TAS are short 15bp template sequences, well conserved among vertebrates and associated with the 3'-ends of arrested D-loop DNA strands (Doda et al., 1981; Fernandez-Silva et al., 2003).

### *Factors required for mtDNA replication*

Although the mode of mtDNA replication is still a controversy, a growing understanding of the key factors involved in mammalian mtDNA replication is beginning to emerge in recent times. MtDNA replication takes place in the mitochondrial matrix and is highly dependent on a number of nuclear-encoded proteins.

### *DNA polymerase $\gamma$ holoenzyme*

Five polymerases are now recognized in *Escherichia coli*, 9 in *Saccharomyces cerevisiae*, and 16 in humans (Goodman, 2002; Hubscher et al., 2002; Shcherbakova



et al., 2003). Based on differences in the primary structure of their catalytic subunits, DNA polymerases are classified into distinct families. The eukaryotic mitochondrial polymerase  $\gamma$  belongs to the family A. The family A members also include the E-coli Pol I, the bacteriophage T7 replicative polymerase, as well as two newly identified polymerases in human cells, Pol  $\theta$  (Seki et al., 2003; Sharief et al., 1999) and Pol  $\nu$  (Marini et al., 2003). The human POL $\gamma$  is a 245kDa heterotrimer, consisting of a catalytic subunit, POL $\gamma$ A, and two identical accessory subunits, POL $\gamma$ B. The catalytic subunit has separate polymerase and 3'-5' exonuclease domains (Yakubovskaya et al., 2006). Two recent studies have clarified the significance of POL $\gamma$ . First, mutations in the catalytic- as well as the accessory subunit of human POL $\gamma$  cause mitochondrial disorders. Secondly, transgenic mice expressing the error-prone form of POL $\gamma$ , lacking the 3'-5' proofreading ability accumulate errors in mtDNA and undergoes accelerated aging (Longley et al., 2006; Trifunovic et al., 2004). The accessory subunit POL $\gamma$ B enhances the catalytic activity and increases the processivity of POL $\gamma$ A. POL $\gamma$ B binds dsDNA longer than 40 bp in a non-specific manner (Carrodeguas et al., 2002) and this dsDNA-binding activity is absolutely required for mtDNA replisome function (Farge et al., 2007).

#### *Mitochondrial single stranded DNA-binding protein*

In order to copy or repair the dsDNA, the double helix must be unwound to reveal the two complementary strands. The need to manipulate DNA in its single-stranded form has given rise to a specialized group of single-stranded DNA-binding proteins. The mtSSB protein has a molecular weight of about 13-16 kDa and has been purified from several species. mtSSB displays sequence similarity to *E. coli* SSB and both proteins form a tetramer in solution (Kaguni, 2004). mtSSB binds cooperatively to DNA with a binding site size of 50-70 nt per tetramer. Deletion of the mtSSB gene (RIM1) in budding yeast causes loss of mitochondrial DNA. The crystal structure of homotetrameric human mtSSB has been solved at 2.4 Å resolution and led to the proposition that ssDNA wraps around the tetrameric mtSSB protein through electropositive channels guided by flexible loops (Van Dyck et al., 1992; Yang et al., 1997).

### *Twinkle-the human mitochondrial DNA helicase*

The TWINKLE gene was originally identified in a search for mutations associated with chromosome 10q24-linked autosomal dominant progressive external ophthalmoplegia. TWINKLE displays structural and sequence similarities to the T7 gp4 helicase/primase (gp4 protein) helicase domain. Multiple sequence alignments revealed that TWINKLE contains motifs typically found among hexameric ring helicases, and belong to the recA/DnaB superfamily. Moreover, TWINKLE was shown to co-localize with mtDNA in nucleoids. The TWINKLE can be structurally divided into three distinct regions: an N-terminal domain with unknown function often referred to as the putative primase domain, a C-terminal helicase domain, and an intervening linker region (about 30 aa). The predicted full-length protein is 684 aa with a molecular mass of 77kDa (cleavable leader peptide 1-42aa). The cDNA amplification revealed the presence of a splice variant of TWINKLE, named TWINKLE. The mRNA of TWINKY encodes a 66 kDa peptide, lacking aa 579-684, and terminating with four unique amino acids (Spelbrink et al., 2001).

A recent study suggests that the N-terminal domain of TWINKLE might be the long sought primase in most eukaryotes except metazoan. The authors identified TWINKLE homologues in representatives from five of the six major eukaryotic assemblage (“supergroups”) and found conserved primase motifs including the zinc-finger in all TWINKLE sequences except those from metazoan (Shutt and Gray, 2006). It is however difficult to determine the role for the N-terminal part of TWINKLE in humans based only on primary sequence analysis. An interesting observation is that there are five different point mutations located in putative primase domain of TWINKLE in adPEO patients indicating an important functional role for this region. So far, 15 different mutations located in TWINKLE are known to co-segregate with the disorder. A majority of the mutations cluster in the intervening linker region believed to be critical for hexamerization (Deschauer et al., 2003; Hudson et al., 2005; Lewis et al., 2002; Spelbrink et al., 2001; Van Goethem et al., 2003). A recent study showed that transgenic mice over-expressing wild type TWINKLE leads to an increase in mtDNA copy number up to three fold in tissues such as heart and muscle (Tyynismaa et al., 2004)

### *Other enzymatic activities required for mtDNA replication*

Two topoisomerases supposedly regulating super-coiling of mtDNA have been identified in human mitochondria, topoisomerase I (top1mt) (Zhang et al., 2001) and

the III $\alpha$  (hTop3 $\alpha$ ) (Wang et al., 2002). More recently, a truncated version of DNA topoisomerase II $\beta$  has been identified in bovine mitochondria, supporting previous observations of a type II activity in the kinetoplast DNA network of protozoan parasites (Low, 2002; Shapiro and Englund, 1995). The DNA end-joining activity on the lagging strand in human mtDNA is mediated by DNA ligase III (Lakshmipathy and Campbell, 1999). Reduction of DNA ligase III expression with RNAi resulted in reduced mtDNA content and the residual mtDNA present had numerous single-stranded nicks (Lakshmipathy and Campbell, 2001). Recent studies indicate that interactions between DNA ligase III and POL $\gamma$  are critical for mtDNA integrity and stability (De and Campbell, 2006).

The different models for mtDNA replication have all in common that they require a primase activity to initiate L-strand replication. A mitochondrial primase activity has been reported but the corresponding protein has not been identified (Vishwanatha and Baril, 1986; Wong and Clayton, 1985). Furthermore, both RNase MRP and RNase H1 have been proposed to be involved in mtDNA replication, with RNase MRP being responsible for primer maturation of the light strand transcript required for leading strand (H-strand) replication. RNase MRP was later demonstrated to have a primarily nuclear localization and its role in mtDNA replication has been questioned (Chang et al., 1987; Pham et al., 2006).

A role for RNase H1 in mammalian mtDNA replication is supported by studies of *Rnaseh1*<sup>-/-</sup> mice, which display a significant decrease in mitochondrial DNA content, leading to apoptotic cell death (Cerritelli et al., 2003). Ribonucleases H activities have mostly been implicated in eliminating short RNA primers used for initiation of lagging strand DNA synthesis. RNase H1 may therefore be required for primer removal at O<sub>H</sub>, and/or at O<sub>L</sub>, or for primer removal in Okazaki fragment synthesis (Gaidamakov et al., 2005). Interestingly, whereas *E. coli* RNase H is a distributive enzyme, mammalian RNases H1 is a processive enzyme able to process long RNA-DNA hybrids. It remains to be determined if the processive action of RNase H1 is essential for digestion of specific intermediates in mtDNA replication.

## **Inheritance and segregation of mtDNA**

The inheritance of the mitochondria genome is believed to be strictly maternal. Upon fertilization of the egg, sperm mitochondria are tagged with ubiquitin and thereby specifically targeted for degradation in the egg cytoplasm (Sutovsky et al., 1999). A rare exception of paternal inheritance of mtDNA was recently described in a patient with mitochondrial myopathy (Schwartz and Vissing, 2002). During oogenesis, mitochondria are randomly segregated and a limited number of mtDNA molecules are amplified and transmitted to the offspring, a so-called bottleneck phenomenon. Due to the random distribution of wild-type and mutant mtDNA during oogenesis a spectrum of heteroplasmy is created across the oocyte population. The transmission rate is dependent on the level of mutant load within the oocyte population i.e. a high mutant load result in a high proportion of affected offspring (Chinnery et al., 1998; Schapira, 2006).

Mitochondrial disease is characterized by the coexistence of wild type and mutant mtDNA to various levels (heteroplasmy). As a consequence of this, the amount of mutated mtDNA has to reach a certain level in order to cause disease, the so called threshold effect. The threshold for disease is lower in tissues that are highly dependent on oxidative metabolism, such as brain, heart, retina, and muscle among others. These kinds of tissues are therefore more susceptible to the effects of pathogenic mutations leading to mitochondrial dysfunction (DiMauro and Schon, 2003). During cell division a random redistribution of mitochondria occurs and this event can in turn change the proportion of mutant versus wild type mtDNA to the daughter cells, which can explain age and tissue-related variability of clinical features in mitochondrial disorders. It has also been speculated that deleted forms of mtDNA has a replicative advantage due to the smaller size genome, which in turn adds to the accumulation of mutated mtDNA in tissues and cells (Diaz et al., 2002; Rotig and Munnich, 2003).

## **Mitochondrial Disorders**

The term “mitochondrial disorders” is commonly used in the medical literature to describe a group of diseases and clinical syndromes related to impaired energy production due to defects in oxidative phosphorylation (OXPHOS) (Zeviani and Di Donato, 2004). At least 1 in 8000 of the general population is affected, making mitochondrial disorders the most common inherited metabolic disease (Chinnery et al.,

2004). Normal mitochondrial function is highly dependent on the complex interaction between the nuclear and mitochondrial genome. Hence, human mitochondrial disorders can be caused by either nuclear or mtDNA gene mutations. Due to this fact, the inheritance of some of the syndromes can be very complicated.

Clinical manifestations are highly variable in mtDNA disorders, which may in part be explained by heteroplasmy, nuclear background and the threshold effect. Moreover, mitochondrial disorders are usually multisystemic since mitochondria are ubiquitous. Since mitochondrial disorders ultimately leads to impaired energy production, tissues which display a high energy demand are those mainly affected, such as brain, heart, liver, and retina. Mitochondrial dysfunction has also been associated with more common diseases such as, Parkinson's disease and Alzheimer's disease, and diabetes (Mathews and Berdanier, 1998; Schapira, 2006).

#### *Mitochondrial DNA mutations*

This group of diseases and syndromes are caused by pathogenic mutations including point mutations, duplications and single deletions within the mtDNA genome itself. These diseases are most often maternally inherited, although some large scale rearrangements normally are sporadic. To date over 150 pathogenic point mutations in the mtDNA that affect protein-encoding genes, tRNAs, and rRNAs have been described and the number is surely increasing. There is no clear link between the site of mutation and the clinical phenotype. One example is mutations found in the tRNA<sup>Leu</sup> (UUR) gene known to be associated with the mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes (MELAS) syndrome, but defects in the tRNA<sup>Leu</sup> gene can cause other syndromes as well (DiMauro and Schon, 2003).

#### *Nuclear gene mutations affecting mtDNA stability*

Studies of defects in the cross-talk between the nuclear and mtDNA genome causing mitochondrial dysfunction have expanded in recent years. Mutations have been found in a number of nuclear encoded factors critical in transcription, replication, repair and maintenance. This group encompasses all diseases caused by nuclear genes that affect mtDNA maintenance and integrity. The diseases thus follow a Mendelian inheritance pattern and are classified as defects of nuclear-mitochondrial intergenomic signaling. Hence, nuclear gene defects secondarily affect the mtDNA stability, causing mutations, deletions or even depletion of the mtDNA (Spinazzola and Zeviani, 2005).

### *Autosomal dominant progressive external ophthalmoplegia*

One of the first findings of an autosomal gene causing mtDNA instability was reported by Zeviani and colleagues in 1989 (Zeviani et al., 1989). They described Italian families with autosomal dominant progressive external ophthalmoplegia (adPEO). They found multiple large-scale deletions of mtDNA in their muscles, and patients suffered from a general muscle weakness. To date at least four gene loci are known to cause PEO; ANT1, TWINKLE, POL $\gamma$ A, and POL $\gamma$ B. However, some adPEO families do not show mutations in any of these genes, indicating further genetic heterogeneity (Kiechl et al., 2004). The ANT1 gene encodes a muscle-heart specific mitochondrial adenine nucleotide translocator located in the inner mitochondrial membrane. PEO follows a Mendelian inheritance and both autosomal dominant and recessive forms of the disorder have been described (Agostino et al., 2003; Longley et al., 2006; Spelbrink et al., 2001; Van Goethem et al., 2002).

One of the main clinical features of adPEO is, as the name implies, progressive weakness of the external eye muscles. As a consequence patients have ptosis and limitations of their eye movements. Other features vary among and even within families but may include sensory ataxia, motor peripheral neuropathy, hearing loss, cataracts, hypogonadism, Parkinsonism, exercise intolerance and severe depression. The onset of the disease is in adulthood and the first symptoms usually arise when patients are between 20-40 years of age. Examination of muscle biopsies typically reveals cytochrome oxidase (COX) negative fibers and ragged-red fibers (RRF). Southern blot analysis of muscle reveals the presence of both wild type and deleted mtDNA genomes. The deletion breakpoints are sometimes flanked by short repeated sequences, which might indicate recombination or slippage-miss-pairing of mtDNA strands during replication (Kawashima et al., 1994; Yuzaki et al., 1989).

To date, there are no known treatments for mtDNA disorders, but as our knowledge increases and suitable animal models are developed new therapeutic inventions might emerge. New strategies for manipulating the level of heteroplasmy are being pursued. The techniques aim at reducing the level of mutant mtDNA by selectively inhibiting the replication of mutant mtDNA by sequence-specific peptide nucleic acids (PNAs) or by removing the mutated mtDNA by restriction enzymes (Chinnery et al., 2004; Chinnery and Turnbull, 2001).

## AIMS OF THIS THESIS

Helicases play essential roles in many biological processes such as, replication, repair and recombination. DNA helicases catalyze unwinding of duplex DNA to single stranded DNA in a process energetically coupled to NTP hydrolysis. With the discovery that mutations and deletions in mtDNA are pathogenic to humans a focus has been set on understanding the mtDNA replication machinery. The general aims of my thesis have been to understand the molecular mechanisms of human mitochondrial DNA replication with a major focus on the recently identified human mitochondrial helicase TWINKLE. By necessity, mechanisms of mtDNA replication have to be investigated in a step-by-step fashion. DNA replication can be seen as the sum of multiple enzymatic reactions; e.g. primer synthesis, DNA unwinding, DNA synthesis, etc. Therefore, a complete understanding of the replication machinery will require that we initially focus our efforts on these discrete reactions, before we combine them into a complete *in vitro* system for mitochondrial DNA replication. The specific aims of each paper were:

**PAPER I:** To characterize the enzymatic activities of TWINKLE *in vitro*.

**PAPER II:** To reconstitute an *in vitro* system for mtDNA replication and study the molecular mechanisms of the mitochondrial replisome.

**PAPER III:** To investigate the molecular basis for adPEO, by characterizing the biochemical consequences of seven different adPEO causing mutations in the linker region of TWINKLE.

**PAPER IV:** To investigate the molecular basis for adPEO, by characterizing the biochemical consequences of four different adPEO causing mutations located in the putative primase domain of TWINKLE.

## RESULTS AND DISCUSSION

### **Biochemical characterization of human TWINKLE *in vitro* (Paper I)**

The TWINKLE gene was originally identified in a search for mutations associated with adPEO. TWINKLE displays primary sequence similarity to the phage T7 gene 4 primase-helicase, but no specific enzymatic activity had been assigned to the protein (Spelbrink et al., 2001). This prompted us to characterize the enzymatic activities of TWINKLE. To this end, recombinant TWINKLE and mtSSB were expressed in insect cells and purified to homogeneity using conventional biochemical techniques. A molecular characterization of TWINKLE was made using a number of biochemical assays *in vitro*. Recombinant TWINKLE migrated as a doublet with an apparent molecular mass of about 70 kDa during SDS-polyacrylamide gel electrophoresis, corresponding well with the predicted mass of 72kDa. TWINKLE was shown to co-purify with a strong ATPase activity supporting predictions that TWINKLE is a Walker-type ATPase. TWINKLE was shown to be an active helicase *in vitro* that could efficiently unwind a 20-bp double-stranded region. TWINKLE displayed a 5' to 3' directionality and required a fork-like structure with both a 5'- and a 3'- single-stranded DNA tails, similar to the DNA fork-like structure requirements described for the gp4 protein (Matson and Richardson, 1983). The unwinding activity was dependent on NTP hydrolysis and addition of the non-hydrolyzable ATP analogue ATP $\gamma$ S inhibited the unwinding activity. The mtSSB protein had a strong stimulatory effect on the unwinding activity of TWINKLE protein. This stimulation was specific because no such effect was observed with *E.coli* SSB. The observed specificity indicated a direct interaction between mtSSB and TWINKLE, similar to what have been demonstrated in other systems, e.g. the herpes simplex virus type 1 helicase-primase which is specifically stimulated by its viral SSB counterpart, ICP8 (Falkenberg et al., 1997; Tanguy Le Gac et al., 1996). Taken together, this study thus demonstrates that TWINKLE is a potent helicase *in vitro* displaying typical features found among hexameric helicases.

### **Reconstitution of the mammalian mtDNA replisome *in vitro* (Paper II)**

It has been proposed that TWINKLE may be the helicase at the mitochondrial DNA replication fork (Spelbrink et al., 2001) but evidence for functional interactions between TWINKLE and POL $\gamma$  had not been presented. We reconstituted for the first



time, a minimal mammalian mitochondrial DNA replisome *in vitro* using highly purified, recombinant proteins. We investigated if human mitochondrial POL $\gamma$ , TWINKLE, and mtSSB could act together at a DNA replication fork, forming a replication machinery resembling the bacteriophage T4 and T7 replisomes. To this end, we used a mini-circle template (a 90-nt oligonucleotide annealed to a 70-nt ssDNA mini-circle). The template formed contained a replication fork for loading the replication machinery, a 50-bp dsDNA region and a free 3'-hydroxyl terminus that could act as a primer for DNA synthesis. POL $\gamma$  on its own could utilize the 3'-hydroxyl terminus of the template, but failed to elongate through the double-stranded region. In a related way, TWINKLE was unable to unwind the mini-circle by itself. In combination, POL $\gamma$  and TWINKLE formed a processive replication machinery, which could use the dsDNA mini-circle template to synthesize ssDNA molecules of about 2 kb. Addition of increasing amounts of mtSSB stimulated the reaction and generated DNA products of about 16-kb, the size of the mammalian mtDNA molecule. The functional interaction between POL $\gamma$  and TWINKLE appeared to be specific, since the mitochondrial POL $\gamma$  could not be replaced by the T7 DNA polymerase or T4 DNA polymerase. Our findings provided the first biochemical evidence that TWINKLE is the helicase at the mtDNA replication fork. The functional interactions between TWINKLE and POL $\gamma$  furthermore explained why mutations in these two proteins could cause the same mitochondrial disorder, adPEO. The data presented in this paper represent an important step in understanding mtDNA replication. Obviously, some limitation exists in utilizing this system *in vitro*. *In vivo*, the mtDNA molecule is negatively super-coiled and covered by TFAM proteins. It is therefore very likely that TFAM as well as other mitochondrial proteins could affect DNA synthesis rate and other aspects of the mtDNA replication process.

### **Analysis of adPEO-causing linker region mutants of TWINKLE. (Paper III)**

We investigated molecular mechanisms by which mutations in the linker region of TWINKLE affect the mtDNA replication. By using the crystal structure of the homologous T7 gp4 protein a molecular model was developed for TWINKLE. We followed previously published protocols and purified a series of mutant proteins in recombinant form, containing the following amino acid changes: R354P, A359T, I367T, V368I, S369P, R374Q, and L381P. The biochemical properties of these mutant proteins were examined in a number of assays and the molecular phenotypes were

explained using the molecular model of TWINKLE. Previous studies of the bacteriophage T7 gp4 protein revealed that hexamerization is a prerequisite for functional helicase and DNA stimulated ATPase activities (Patel et al., 1994). Similar to TWINKLE, the gp4 protein contains a 26 aa linker region that is critical for hexamer formation and hence helicase activity (Guo et al., 1999; Lee and Richardson, 2004). We characterized the mutant forms of TWINKLE for their ability to hexamerize under native conditions with size exclusion chromatography. Two out of six mutants, I367T and R374Q, failed to form stable hexamers and were eluted as monomers. The two monomers I367T, and R374Q, demonstrated severely reduced activities in all enzymatic assays performed, i.e. ATP hydrolysis, DNA unwinding, binding to ssDNA, and DNA synthesis on a mini-circle template.

The S369P and L381P mutants could form hexamers, but displayed severely reduced levels of ATP hydrolysis, unwinding, and ssDNA binding activities. It is known in other studies that ssDNA binding is strongly stimulated by the presence of ATP (Hingorani and Patel, 1993). Our structural model suggest that the L381P interacts specifically with residues in the conserved H1a motif of the neighboring subunit, known to be involved in ATP binding and we therefore believe that this mutant may disrupt the ATP binding or hydrolysis ability of the TWINKLE protein. In a similar fashion the S369P mutant had reduced ATP hydrolysis activities, reaching only about 30% of wt activities as well as displaying severely reduced ssDNA binding activities. Taken together, these results indicated that S369P also had similar biochemical defects. The A359T mutant almost reached wt activities in all assays tested and the protein could support POL $\gamma$ -dependent DNA synthesis on a mini-circle template although with lower extend (50%) compared to wild-type TWINKLE. The last mutant investigated, V368I, behaved as the WT TWINKLE protein all assays tested, supporting the notion that this is a polymorphic mutation without phenotypic consequences (Arenas et al., 2003).

#### **Analysis of N-terminal adPEO mutants of TWINKLE (paper IV)**

We wanted to address the molecular basis for adPEO mutations located in the N-terminal domain of TWINKLE. The N-terminal part (aa 43-248) of TWINKLE corresponds to a large portion of the protein, but the molecular function of this region remains unknown. It would be tempting to speculate that this region contains the long sought primase activity in human mitochondria, since a number of conserved primase motifs are located in the first half of the human TWINKLE gene (Shutt and Gray,

2006). However, we have not been able to detect any primase activities (unpublished data) and neither have there been any reports of a primase activity of TWINKLE to date. Two major arguments against a role of TWINKLE as a primase is the lack of the zinc-binding domain critical for DNA binding and the absence of essential amino acids required for nucleotide polymerization (Kusakabe et al., 1999; Powers and Griep, 1999; Shutt and Gray, 2006).

A set of 4 adPEO-associated mutations, W315L, K319T, R334Q, and P335L, were generated and their biochemical properties were tested with a number of assays. The mutant proteins could all form stable hexamers *in vitro*. Single-stranded DNA binding activities were addressed in the presence of ATP since TWINKLE requires ATP or a non-hydrolyzing ATP analog to stably interact with ssDNA (Farge et al, submitted). The R334Q bound to ssDNA with a similar efficiency as wt TWINKLE. K319T and P335L bound ssDNA with a severely reduced capacity, whereas W315L displayed no ssDNA binding activity at all. The ATPase activities of the mutants were analyzed with and without ssDNA. By investigating the ATPase activity in the presence of ssDNA one will get an indirect measurement of each proteins ability to move on ssDNA and hence their unwinding ability on dsDNA. By analyzing ATP hydrolysis in the absence of ssDNA one can get information about the intrinsic ability of the helicase to bind and hydrolyze ATP. Wt TWINKLE was stimulated 2 fold in the presence of ssDNA displaying a rather high intrinsic ability for ATP binding and hydrolysis. In the absence of ssDNA, all four mutants had severely reduced ability to hydrolyze ATP (4 - 16% relative the wt TWINKLE protein). Interestingly, when ssDNA is present in the ATPase assay the K319T and R334Q hydrolyze ATP with nearly the same efficiency as wt protein. The deficiency in ATP hydrolysis observed in this study by the four mutants was not expected, since the position of typical walker A motifs important for NTP binding are located in the second half of the protein, in the helicase domain of TWINKLE.

We next investigated the dsDNA unwinding ability of the four adPEO-causing mutants and compared them to wt activities. The K319T mutant was able to unwind dsDNA as efficiently as wt TWINKLE, meanwhile the R334Q, P335L and W315L displayed reduced activities. The mini-circle assay demonstrated that only the R334Q mutant is active together with POL $\gamma$  and support DNA synthesis. The drastic phenotype associated with K319T was not expected since this protein unwinds dsDNA

as efficient as wt TWINKLE although with a slower initial activity rate. The severe defects of all four mutants analyzed in this study indicate that the N-terminal part of TWINKLE is a crucial region and the structural properties of this region influence a number of biochemical activities known to be located in more distal parts of the protein. The structural model of TWINKLE reveals that the adPEO causing amino acid substitutions W315L, K319T, R334Q, and P335L are all located in a region required to bind single-stranded DNA. We propose a model in which these adPEO causing mutations impair the interplay between single-stranded DNA binding and ATP hydrolysis, which is an essential element of the catalytic cycle of related hexameric helicases.

## CONCLUDING REMARKS

Work to elucidate the molecular mechanism of mtDNA replication has been hampered by the lack of a reconstituted system that can faithfully reproduce this fundamental process *in vitro*. In our efforts to establish such a system, we characterized the TWINKLE helicase to understand its functional role in human mtDNA replication. We found that TWINKLE was an active helicase with 5' to 3' directionality, which could be specifically stimulated by mtSSB. Having established the basic activities associated with TWINKLE, we attempted to reconstitute leading strand DNA synthesis *in vitro*. Previous work had established that POL $\gamma$  is active in recombinant form and we now attempted to combine the polymerase with TWINKLE and mtSSB to form a mitochondrial replication machinery, a replisome. To this end, we used a mini-circle duplex DNA template and found that POL $\gamma$  and TWINKLE in combination could utilize this template to synthesize ssDNA molecules of about 2 kb. MtSSB stimulated the reaction, generating DNA products of about 16-kb, the size of the mammalian mtDNA molecule.

We could utilize the *in vitro* replication system to investigate the molecular mechanism by which mutations in TWINKLE disturb mtDNA replication and cause adPEO. We performed a detailed analysis of eleven different adPEO-causing mutations, seven in the linker region of TWINKLE and four in the N-terminal primase-like domain. Our analyses revealed different molecular phenotypes, with distinct consequences for multimerization, ATPase activity, DNA helicase activity, and ability to support mtDNA synthesis *in vitro*. The distinct molecular phenotypes could be interpreted using a structural model of TWINKLE that was based on extensive primary sequence similarities with the gp4 protein. These studies provided a molecular understanding of adPEO and demonstrated that knowledge of the bacteriophage T7 DNA replication machinery may be key to understanding molecular and phenotypic consequences of mutations in the mitochondrial DNA replication apparatus.

## PERSPECTIVES

The result presented in this thesis may now be continued in a couple of different directions. The reconstituted *in vitro* system can be used to examine the mechanism of mtDNA replication. What are the protein-protein and protein-DNA interactions that coordinate movement of POL $\gamma$  and TWINKLE on the mtDNA replication fork? Can we use the *in vitro* system to search for additional factors in mitochondrial extracts that can stimulate or repress the mtDNA replication? We have reconstituted leading strand DNA synthesis, but yet unidentified replication factors required for lagging strand DNA synthesis to occur? What are the mechanisms of lagging-strand DNA synthesis and how is it coupled to leading strand DNA synthesis? What is the frequency of priming events and how are these events triggered? How is the replisome assembled at the mitochondrial origins of replication? How is initiation of mtDNA replication at O<sub>H</sub> coupled to the transcription system?

Investigations into these and related questions will provide more detailed information about mtDNA replication and may also help us to identify drug targets for treatment of mitochondrial disorders.

## ACKNOWLEDGEMENTS

This thesis has been carried out in the Department of Laboratory Medicine (as well as Dept. of Medical Nutrition), at the Karolinska Institute. It has been four years of great experiences both scientific and personal ones. I had a daughter half way through my thesis work and that obviously changed my life in a profound and positive way. I have also met many nice and friendly people in and outside of the lab here in Stockholm that I would like to mention. Also, I would like to take this opportunity to express my sincere gratitude for the support and encouragement I have received from a number of people that made this thesis work possible.

I would like to thank the following persons:

My supervisor **Maria Falkenberg**, for accepting me as your PhD student and introducing me to the field of mitochondrial replication with a never-ending enthusiasm. I am grateful for your good advice, for your friendship, support and guidance over the years. You are an excellent supervisor, always taking time to discuss both science as well as life in general. Thanks for pushing me forward when I needed it so that I could finish in this short time. I will especially cherish some really fun moments outside of work such as the great time we had in Colorado when we went skiing etc.

My co-supervisor, **Claes Gustafsson**, for great scientific advices, your patience and belief in me. I especially appreciate your contagious enthusiasm and positive attitude towards life in general. I am thankful for all the help and support I received over the years such as reading through this thesis and reading my papers before printing, just to mention some important ones.

My co-supervisor **Nils-Göran Larsson**, for your generosity, inspiring ways, and dedication to science and guiding me by providing me and others with excellent lectures. Thank you for taking me onboard and believing in me, as you took part in recruiting process. I also appreciate that you allowed me to come along on conferences with some of your group members. I think it was very nice, such as Holland where I got a chance to know you and your lab members a bit better.

For members in my lab: **Teresa Holmlund**. For your friendship and help over the years. Small favours that added up in the end to something big. Thanks! I think your organized ways inspired me to better myself as well as bring more order into the lab. I am pretty sure we will see more of each other in the future and I am glad over that.

**Yonghong**, for your happy mood and taking time for scientific and non-scientific discussions. It has been great getting to know you and your husband **Xufeng** also outside of lab. You are both very nice people.

**Mina** (I consider you a lab member), **Geraldine**, **Jordi**, **Nelli** and **Martina** for helping me with scientific advice and support. You are all very nice and I am glad I had the opportunity to be part of your lab.

Members in Claes lab for your help and support, helping me find things I wanted: The friendly Russian trio; **Olga**, **Vera**, and **Julia. Emanuella**, whom always is nice and

friendly no matter if you are busy yourself, and **Tomas**-you are a very funny guy that have made me smile more than once. **Olivera**, for your help, but also for your friendship over the years. I think it was a really great that we got pregnant at the same period in life and got a chance to meet more outside of work. **Fariba**, for helping me from time to time in the cell culture room, among other things. I really enjoy your calm manners and happy mood, you can really reduce the stress state I am in by just talking to me.

Other members of Anna's and Nils-Göran's lab. My nice and friendly, bench comrade in the writing room, **Yunjian**, it has been fun having biochemical discussions and talking about life in general. Other nice people in the writing room: **Yolanda**, **Ivana**, **Mina**, **Mügen**, **Metodi**, **Marjan**, and **Jordi** it has been great fun to sit in the same room, listening and sometimes taking part in the lively discussions taking place in there.

**Lene**, **Zekiye**, **Jim**, **Chris**, **Anna**, **Ivan**, **Daniel**, **Fredrik**, **Camilla**, **Mia**, **Sophie**, **Chan Bae**, **Xioshan**, **Benedetta**, and **Nicola** for nice lunch breaks, and for helping me in some ways I am sure.

Some great friends here in Stockholm who made me stay sane during stressful times, keeping my spirit up. Between work and being with my family, I wish the day had more hours so I could see you all more frequent than now a days...

**Pia Kotokorpi**, whom I met in science and "lured" into becoming a PhD student. We have had many fun times together and I hope for many more. **Ulrika Marklund** over at Solna campus, my friend from Umeå whom I have had great dinners with discussing both science and what the future holds. Soon its your turn to write your thesis, count on me if you need help. **Alexandra T**, Kungholmen for being a good friend. I just heard the news, congratulations on your daughter!! **Ulrika**, **Ulf**, and **Thea**. It has been great to have you here in Stockholm spending many weekends together always doing something fun. **Axel** and **Anna**, for your friendship and taking our mind of work doing fun things together. **Linda** and **Adam with family**, it has been great getting to know some new friendly people living so close. **Camilla**, **Martin** and **Hugo** thanks for your friendship and generosity. Past member of the lab **Jenny Beve**, who is a good friend and listener. I have spent many fun lunch hours with you during the years and I missed you when you left.

**My family** up north in the lovely city of Luleå, your support and love mean the world to me. For always being there for me, I am so glad we keep in touch as much as we do and you all make it so easy for me to miss you. It is always lovely to come home and visit and I feel totally refreshed and full of energy every time I have been up visiting.

Last but not least, my own family, my partner **Björn** and my daughter **Tyra**. I love you both very much and life would be meaningless without the two of you. Soon one more member will be added to our little trio which is very exciting. Björn, you have helped me in so many ways over the years and I sincerely appreciate the, love, support and faith you have in me. I couldn't have done it without you.



## REFERENCES

- Abdel-Monem, M. and Hoffmann-Berling, H. (1976) Enzymic unwinding of DNA. 1. Purification and characterization of a DNA-dependent ATPase from *Escherichia coli*. *Eur J Biochem*, **65**, 431-440.
- Agostino, A., Valletta, L., Chinnery, P.F., Ferrari, G., Carrara, F., Taylor, R.W., Schaefer, A.M., Turnbull, D.M., Tiranti, V. and Zeviani, M. (2003) Mutations of ANT1, Twinkle, and POLG1 in sporadic progressive external ophthalmoplegia (PEO). *Neurology*, **60**, 1354-1356.
- Alam, T.I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., Takio, K., Hamasaki, N. and Kang, D. (2003) Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res*, **31**, 1640-1645.
- Albring, M., Griffith, J. and Attardi, G. (1977) Association of a protein structure of probable membrane derivation with HeLa cell mitochondrial DNA near its origin of replication. *Proc Natl Acad Sci U S A*, **74**, 1348-1352.
- Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J., Staden, R. and Young, I.G. (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457-465.
- Andersson, S.G., Zomorodipour, A., Andersson, J.O., Sicheritz-Ponten, T., Alsmark, U.C., Podowski, R.M., Naslund, A.K., Eriksson, A.S., Winkler, H.H. and Kurland, C.G. (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature*, **396**, 133-140.
- Arenas, J., Briem, E., Dahl, H., Hutchison, W., Lewis, S., Martin, M.A., Spelbrink, H., Tiranti, V., Jacobs, H. and Zeviani, M. (2003) The V368i mutation in Twinkle does not segregate with AdPEO. *Ann Neurol*, **53**, 278.
- Asin-Cayuela, J., Schwend, T., Farge, G. and Gustafsson, C.M. (2005) The human mitochondrial transcription termination factor (mTERF) is fully active in vitro in the non-phosphorylated form. *J Biol Chem*, **280**, 25499-25505.
- Benkovic, S.J., Valentine, A.M. and Salinas, F. (2001) Replisome-mediated DNA replication. *Annu Rev Biochem*, **70**, 181-208.
- Berk, A.J. and Clayton, D.A. (1974) Mechanism of mitochondrial DNA replication in mouse L-cells: asynchronous replication of strands, segregation of circular daughter molecules, aspects of topology and turnover of an initiation sequence. *J Mol Biol*, **86**, 801-824.
- Berk, A.J. and Clayton, D.A. (1976) Mechanism of mitochondrial DNA replication in mouse L-cells: topology of circular daughter molecules and dynamics of catenated oligomer formation. *J Mol Biol*, **100**, 85-92.
- Bernstein, J.A. and Richardson, C.C. (1989) Characterization of the helicase and primase activities of the 63-kDa component of the bacteriophage T7 gene 4 protein. *J Biol Chem*, **264**, 13066-13073.
- Bogenhagen, D. and Clayton, D.A. (1977) Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell*, **11**, 719-727.
- Bogenhagen, D.F., Applegate, E.F. and Yoza, B.K. (1984) Identification of a promoter for transcription of the heavy strand of human mtDNA: in vitro transcription and deletion mutagenesis. *Cell*, **36**, 1105-1113.

- Bogenhagen, D.F. and Clayton, D.A. (2003a) Concluding remarks: The mitochondrial DNA replication bubble has not burst. *Trends Biochem Sci*, **28**, 404-405.
- Bogenhagen, D.F. and Clayton, D.A. (2003b) The mitochondrial DNA replication bubble has not burst. *Trends Biochem Sci*, **28**, 357-360.
- Bowmaker, M., Yang, M.Y., Yasukawa, T., Reyes, A., Jacobs, H.T., Huberman, J.A. and Holt, I.J. (2003) Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. *J Biol Chem*, **278**, 50961-50969.
- Brown, T.A., Cecconi, C., Tkachuk, A.N., Bustamante, C. and Clayton, D.A. (2005) Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. *Genes Dev*, **19**, 2466-2476.
- Bujalowski, W., Klonowska, M.M. and Jezewska, M.J. (1994) Oligomeric structure of Escherichia coli primary replicative helicase DnaB protein. *J Biol Chem*, **269**, 31350-31358.
- Caron, F., Jacq, C. and Rouviere-Yaniv, J. (1979) Characterization of a histone-like protein extracted from yeast mitochondria. *Proc Natl Acad Sci U S A*, **76**, 4265-4269.
- Carrodeguas, J.A., Pinz, K.G. and Bogenhagen, D.F. (2002) DNA binding properties of human pol gammaB. *J Biol Chem*, **277**, 50008-50014.
- Cerritelli, S.M., Frolova, E.G., Feng, C., Grinberg, A., Love, P.E. and Crouch, R.J. (2003) Failure to produce mitochondrial DNA results in embryonic lethality in Rnaseh1 null mice. *Mol Cell*, **11**, 807-815.
- Chang, D.D. and Clayton, D.A. (1985) Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc Natl Acad Sci U S A*, **82**, 351-355.
- Chang, D.D., Fisher, R.P. and Clayton, D.A. (1987) Roles for a promoter and RNA processing in the synthesis of mitochondrial displacement-loop strands. *Biochim Biophys Acta*, **909**, 85-91.
- Chen, X.J., Wang, X., Kaufman, B.A. and Butow, R.A. (2005) Aconitase couples metabolic regulation to mitochondrial DNA maintenance. *Science*, **307**, 714-717.
- Chinnery, P.F., DiMauro, S., Shanske, S., Schon, E.A., Zeviani, M., Mariotti, C., Carrara, F., Lombes, A., Laforet, P., Ogier, H., Jaksch, M., Lochmuller, H., Horvath, R., Deschauer, M., Thorburn, D.R., Bindoff, L.A., Poulton, J., Taylor, R.W., Matthews, J.N. and Turnbull, D.M. (2004) Risk of developing a mitochondrial DNA deletion disorder. *Lancet*, **364**, 592-596.
- Chinnery, P.F., Howell, N., Lightowlers, R.N. and Turnbull, D.M. (1998) MELAS and MERRF. The relationship between maternal mutation load and the frequency of clinically affected offspring. *Brain*, **121** ( Pt 10), 1889-1894.
- Chinnery, P.F. and Turnbull, D.M. (2001) Epidemiology and treatment of mitochondrial disorders. *Am J Med Genet*, **106**, 94-101.
- Clayton, D.A. (1982) Replication of animal mitochondrial DNA. *Cell*, **28**, 693-705.
- Clayton, D.A. (2003) Mitochondrial DNA replication: what we know. *IUBMB Life*, **55**, 213-217.
- De, A. and Campbell, C. (2006) A novel interaction between DNA ligase III and DNA polymerase gamma plays an essential role in mitochondrial DNA stability. *Biochem J*.
- Debyser, Z., Tabor, S. and Richardson, C.C. (1994) Coordination of leading and lagging strand DNA synthesis at the replication fork of bacteriophage T7. *Cell*, **77**, 157-166.

- Deschauer, M., Kiefer, R., Blakely, E.L., He, L., Zierz, S., Turnbull, D.M. and Taylor, R.W. (2003) A novel Twinkle gene mutation in autosomal dominant progressive external ophthalmoplegia. *Neuromuscul Disord*, **13**, 568-572.
- Diaz, F., Bayona-Bafaluy, M.P., Rana, M., Mora, M., Hao, H. and Moraes, C.T. (2002) Human mitochondrial DNA with large deletions repopulates organelles faster than full-length genomes under relaxed copy number control. *Nucleic Acids Res*, **30**, 4626-4633.
- DiMauro, S. and Schon, E.A. (2003) Mitochondrial respiratory-chain diseases. *N Engl J Med*, **348**, 2656-2668.
- Doda, J.N., Wright, C.T. and Clayton, D.A. (1981) Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. *Proc Natl Acad Sci U S A*, **78**, 6116-6120.
- Dong, F., Gogol, E.P. and von Hippel, P.H. (1995) The phage T4-coded DNA replication helicase (gp41) forms a hexamer upon activation by nucleoside triphosphate. *J Biol Chem*, **270**, 7462-7473.
- Donmez, I. and Patel, S.S. (2006) Mechanisms of a ring shaped helicase. *Nucleic Acids Res*, **34**, 4216-4224.
- Dunn, J.J. and Studier, F.W. (1983) Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J Mol Biol*, **166**, 477-535.
- Egelman, E.H., Yu, X., Wild, R., Hingorani, M.M. and Patel, S.S. (1995) Bacteriophage T7 helicase/primase proteins form rings around single-stranded DNA that suggest a general structure for hexameric helicases. *Proc Natl Acad Sci U S A*, **92**, 3869-3873.
- Falkenberg, M., Bushnell, D.A., Elias, P. and Lehman, I.R. (1997) The UL8 subunit of the heterotrimeric herpes simplex virus type 1 helicase-primase is required for the unwinding of single strand DNA-binding protein (ICP8)-coated DNA substrates. *J Biol Chem*, **272**, 22766-22770.
- Falkenberg, M., Gaspari, M., Rantanen, A., Trifunovic, A., Larsson, N.G. and Gustafsson, C.M. (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat Genet*, **31**, 289-294.
- Farge, G., Pham, X.H., Holmlund, T., Holmlund, T., Khorostov, I. and Falkenberg, M. (2007) The accessory subunit B of DNA polymerase gamma is required for mitochondrial replisome function. *Nucleic Acids Res*, **In press**.
- Fernandez-Silva, P., Enriquez, J.A. and Montoya, J. (2003) Replication and transcription of mammalian mitochondrial DNA. *Exp Physiol*, **88**, 41-56.
- Fisher, R.P., Lisowsky, T., Parisi, M.A. and Clayton, D.A. (1992) DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. *J Biol Chem*, **267**, 3358-3367.
- Frick, D.N., Baradaran, K. and Richardson, C.C. (1998) An N-terminal fragment of the gene 4 helicase/primase of bacteriophage T7 retains primase activity in the absence of helicase activity. *Proc Natl Acad Sci U S A*, **95**, 7957-7962.
- Gaidamakov, S.A., Gorshkova, II, Schuck, P., Steinbach, P.J., Yamada, H., Crouch, R.J. and Cerritelli, S.M. (2005) Eukaryotic RNases H1 act processively by interactions through the duplex RNA-binding domain. *Nucleic Acids Res*, **33**, 2166-2175.
- Garrido, N., Griparic, L., Jokitalo, E., Wartiovaara, J., van der Bliek, A.M. and Spelbrink, J.N. (2003) Composition and dynamics of human mitochondrial nucleoids. *Mol Biol Cell*, **14**, 1583-1596.
- Goodman, M.F. (2002) Error-prone repair DNA polymerases in prokaryotes and eukaryotes. *Annu Rev Biochem*, **71**, 17-50.
- Gray, M.W., Burger, G. and Lang, B.F. (1999) Mitochondrial evolution. *Science*, **283**, 1476-1481.

- Guo, S., Tabor, S. and Richardson, C.C. (1999) The linker region between the helicase and primase domains of the bacteriophage T7 gene 4 protein is critical for hexamer formation. *J Biol Chem*, **274**, 30303-30309.
- Hamdan, S.M., Marintcheva, B., Cook, T., Lee, S.J., Tabor, S. and Richardson, C.C. (2005) A unique loop in T7 DNA polymerase mediates the binding of helicase-primase, DNA binding protein, and processivity factor. *Proc Natl Acad Sci U S A*, **102**, 5096-5101.
- Hingorani, M.M. and Patel, S.S. (1993) Interactions of bacteriophage T7 DNA primase/helicase protein with single-stranded and double-stranded DNAs. *Biochemistry*, **32**, 12478-12487.
- Hixson, J.E., Wong, T.W. and Clayton, D.A. (1986) Both the conserved stem-loop and divergent 5'-flanking sequences are required for initiation at the human mitochondrial origin of light-strand DNA replication. *J Biol Chem*, **261**, 2384-2390.
- Hobbs, A.E., Srinivasan, M., McCaffery, J.M. and Jensen, R.E. (2001) Mmm1p, a mitochondrial outer membrane protein, is connected to mitochondrial DNA (mtDNA) nucleoids and required for mtDNA stability. *J Cell Biol*, **152**, 401-410.
- Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1988) Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature*, **331**, 717-719.
- Holt, I.J. and Jacobs, H.T. (2003) Response: The mitochondrial DNA replication bubble has not burst. *Trends Biochem Sci*, **28**, 355-356.
- Holt, I.J., Lorimer, H.E. and Jacobs, H.T. (2000) Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell*, **100**, 515-524.
- Hubscher, U., Maga, G. and Spadari, S. (2002) Eukaryotic DNA polymerases. *Annu Rev Biochem*, **71**, 133-163.
- Hudson, G., Deschauer, M., Busse, K., Zierz, S. and Chinnery, P.F. (2005) Sensory ataxic neuropathy due to a novel C10orf2 mutation with probable germline mosaicism. *Neurology*, **64**, 371-373.
- Iborra, F.J., Kimura, H. and Cook, P.R. (2004) The functional organization of mitochondrial genomes in human cells. *BMC Biol*, **2**, 9.
- Jeong, Y.J., Levin, M.K. and Patel, S.S. (2004) The DNA-unwinding mechanism of the ring helicase of bacteriophage T7. *Proc Natl Acad Sci U S A*, **101**, 7264-7269.
- Kaguni, L.S. (2004) DNA polymerase gamma, the mitochondrial replicase. *Annu Rev Biochem*, **73**, 293-320.
- Kasamatsu, H., Robberson, D.L. and Vinograd, J. (1971) A novel closed-circular mitochondrial DNA with properties of a replicating intermediate. *Proc Natl Acad Sci U S A*, **68**, 2252-2257.
- Kaufman, B.A., Newman, S.M., Hallberg, R.L., Slaughter, C.A., Perlman, P.S. and Butow, R.A. (2000) In organello formaldehyde crosslinking of proteins to mtDNA: identification of bifunctional proteins. *Proc Natl Acad Sci U S A*, **97**, 7772-7777.
- Kawashima, S., Ohta, S., Kagawa, Y., Yoshida, M. and Nishizawa, M. (1994) Widespread tissue distribution of multiple mitochondrial DNA deletions in familial mitochondrial myopathy. *Muscle Nerve*, **17**, 741-746.
- Kiechl, S., Horvath, R., Luoma, P., Kiechl-Kohlendorfer, U., Wallacher-Scholz, B., Stucka, R., Thaler, C., Wanschitz, J., Suomalainen, A., Jaksch, M. and Willeit, J. (2004) Two families with autosomal dominant progressive external ophthalmoplegia. *J Neurol Neurosurg Psychiatry*, **75**, 1125-1128.

- Kim, Y.T. and Richardson, C.C. (1994) Acidic carboxyl-terminal domain of gene 2.5 protein of bacteriophage T7 is essential for protein-protein interactions. *J Biol Chem*, **269**, 5270-5278.
- Kim, Y.T., Tabor, S., Bortner, C., Griffith, J.D. and Richardson, C.C. (1992) Purification and characterization of the bacteriophage T7 gene 2.5 protein. A single-stranded DNA-binding protein. *J Biol Chem*, **267**, 15022-15031.
- Krause, F., Reifschneider, N.H., Vocke, D., Seelert, H., Rexroth, S. and Dencher, N.A. (2004) "Respirasome"-like supercomplexes in green leaf mitochondria of spinach. *J Biol Chem*, **279**, 48369-48375.
- Kusakabe, T., Hine, A.V., Hyberts, S.G. and Richardson, C.C. (1999) The Cys4 zinc finger of bacteriophage T7 primase in sequence-specific single-stranded DNA recognition. *Proc Natl Acad Sci U S A*, **96**, 4295-4300.
- Lakshmipathy, U. and Campbell, C. (1999) The human DNA ligase III gene encodes nuclear and mitochondrial proteins. *Mol Cell Biol*, **19**, 3869-3876.
- Lakshmipathy, U. and Campbell, C. (2001) Antisense-mediated decrease in DNA ligase III expression results in reduced mitochondrial DNA integrity. *Nucleic Acids Res*, **29**, 668-676.
- Larsson, N.G. and Clayton, D.A. (1995) Molecular genetic aspects of human mitochondrial disorders. *Annu Rev Genet*, **29**, 151-178.
- Larsson, N.G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S. and Clayton, D.A. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet*, **18**, 231-236.
- Lecrenier, N., Van Der Bruggen, P. and Foury, F. (1997) Mitochondrial DNA polymerases from yeast to man: a new family of polymerases. *Gene*, **185**, 147-152.
- Lee, J., Chastain, P.D., 2nd, Kusakabe, T., Griffith, J.D. and Richardson, C.C. (1998) Coordinated leading and lagging strand DNA synthesis on a minicircular template. *Mol Cell*, **1**, 1001-1010.
- Lee, S.J. and Richardson, C.C. (2004) The linker region between the helicase and primase domains of the gene 4 protein of bacteriophage T7. Role in helicase conformation and activity. *J Biol Chem*, **279**, 23384-23393.
- Lewis, S., Hutchison, W., Thyagarajan, D. and Dahl, H.H. (2002) Clinical and molecular features of adPEO due to mutations in the Twinkle gene. *J Neurol Sci*, **201**, 39-44.
- Lightowlers, R.N., Chinnery, P.F., Turnbull, D.M. and Howell, N. (1997) Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. *Trends Genet*, **13**, 450-455.
- Longley, M.J., Clark, S., Yu Wai Man, C., Hudson, G., Durham, S.E., Taylor, R.W., Nightingale, S., Turnbull, D.M., Copeland, W.C. and Chinnery, P.F. (2006) Mutant POLG2 disrupts DNA polymerase gamma subunits and causes progressive external ophthalmoplegia. *Am J Hum Genet*, **78**, 1026-1034.
- Low, R.L. (2002) Isolation of DNA topoisomerases I and II activities from mitochondria of mammalian heart. *Methods Mol Biol*, **197**, 317-329.
- Luft, R., Ikkos, D., Palmieri, G., Ernster, L. and Afzelius, B. (1962) A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical, and morphological study. *J Clin Invest*, **41**, 1776-1804.
- Madsen, C.S., Ghivizzani, S.C. and Hauswirth, W.W. (1993) Protein binding to a single termination-associated sequence in the mitochondrial DNA D-loop region. *Mol Cell Biol*, **13**, 2162-2171.

- Malka, F., Lombes, A. and Rojo, M. (2006) Organization, dynamics and transmission of mitochondrial DNA: focus on vertebrate nucleoids. *Biochim Biophys Acta*, **1763**, 463-472.
- Marini, F., Kim, N., Schuffert, A. and Wood, R.D. (2003) POLN, a nuclear PolA family DNA polymerase homologous to the DNA cross-link sensitivity protein Mus308. *J Biol Chem*, **278**, 32014-32019.
- Masters, B.S., Stohl, L.L. and Clayton, D.A. (1987) Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell*, **51**, 89-99.
- Mathews, C.E. and Berdanier, C.D. (1998) Noninsulin-dependent diabetes mellitus as a mitochondrial genomic disease. *Proc Soc Exp Biol Med*, **219**, 97-108.
- Matson, S.W. and Richardson, C.C. (1983) DNA-dependent nucleoside 5'-triphosphatase activity of the gene 4 protein of bacteriophage T7. *J Biol Chem*, **258**, 14009-14016.
- McCulloch, V., Seidel-Rogol, B.L. and Shadel, G.S. (2002) A human mitochondrial transcription factor is related to RNA adenine methyltransferases and binds S-adenosylmethionine. *Mol Cell Biol*, **22**, 1116-1125.
- Mendelman, L.V. and Richardson, C.C. (1991) Requirements for primer synthesis by bacteriophage T7 63-kDa gene 4 protein. Roles of template sequence and T7 56-kDa gene 4 protein. *J Biol Chem*, **266**, 23240-23250.
- Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*, **191**, 144-148.
- Montoya, J., Christianson, T., Levens, D., Rabinowitz, M. and Attardi, G. (1982) Identification of initiation sites for heavy-strand and light-strand transcription in human mitochondrial DNA. *Proc Natl Acad Sci U S A*, **79**, 7195-7199.
- Montoya, J., Gaines, G.L. and Attardi, G. (1983) The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell*, **34**, 151-159.
- Morris, C.F., Sinha, N.K. and Alberts, B.M. (1975) Reconstruction of bacteriophage T4 DNA replication apparatus from purified components: rolling circle replication following de novo chain initiation on a single-stranded circular DNA template. *Proc Natl Acad Sci U S A*, **72**, 4800-4804.
- Nass, M.M. and Nass, S. (1963) Intramitochondrial Fibers with DNA Characteristics. I. Fixation and Electron Staining Reactions. *J Cell Biol*, **19**, 593-611.
- Notarnicola, S.M., Mulcahy, H.L., Lee, J. and Richardson, C.C. (1997) The acidic carboxyl terminus of the bacteriophage T7 gene 4 helicase/primase interacts with T7 DNA polymerase. *J Biol Chem*, **272**, 18425-18433.
- Park, K., Debyser, Z., Tabor, S., Richardson, C.C. and Griffith, J.D. (1998) Formation of a DNA loop at the replication fork generated by bacteriophage T7 replication proteins. *J Biol Chem*, **273**, 5260-5270.
- Patel, S.S., Hingorani, M.M. and Ng, W.M. (1994) The K318A mutant of bacteriophage T7 DNA primase-helicase protein is deficient in helicase but not primase activity and inhibits primase-helicase protein wild-type activities by heterooligomer formation. *Biochemistry*, **33**, 7857-7868.
- Patel, S.S. and Picha, K.M. (2000) Structure and function of hexameric helicases. *Annu Rev Biochem*, **69**, 651-697.
- Pham, X.H., Farge, G., Shi, Y., Gaspari, M., Gustafsson, C.M. and Falkenberg, M. (2006) Conserved sequence box II directs transcription termination and primer formation in mitochondria. *J Biol Chem*, **281**, 24647-24652.
- Powers, L. and Griep, M.A. (1999) Escherichia coli primase zinc is sensitive to substrate and cofactor binding. *Biochemistry*, **38**, 7413-7420.

- Racker, E. (1980) From Pasteur to Mitchell: a hundred years of bioenergetics. *Fed Proc*, **39**, 210-215.
- Richardson, C.C. (1983) Bacteriophage T7: minimal requirements for the replication of a duplex DNA molecule. *Cell*, **33**, 315-317.
- Robberson, D.L. and Clayton, D.A. (1972) Replication of mitochondrial DNA in mouse L cells and their thymidine kinase - derivatives: displacement replication on a covalently-closed circular template. *Proc Natl Acad Sci U S A*, **69**, 3810-3814.
- Rotig, A. and Munnich, A. (2003) Genetic features of mitochondrial respiratory chain disorders. *J Am Soc Nephrol*, **14**, 2995-3007.
- Rousvoal, S., Oudot, M., Fontaine, J., Kloareg, B. and Goer, S.L. (1998) Witnessing the evolution of transcription in mitochondria: the mitochondrial genome of the primitive brown alga *Pylaiella littoralis* (L.) Kjellm. Encodes a T7-like RNA polymerase. *J Mol Biol*, **277**, 1047-1057.
- Sawaya, M.R., Guo, S., Tabor, S., Richardson, C.C. and Ellenberger, T. (1999) Crystal structure of the helicase domain from the replicative helicase-primase of bacteriophage T7. *Cell*, **99**, 167-177.
- Schafer, E., Seelert, H., Reifschneider, N.H., Krause, F., Dencher, N.A. and Vonck, J. (2006) Architecture of active mammalian respiratory chain supercomplexes. *J Biol Chem*, **281**, 15370-15375.
- Schagger, H. and Pfeiffer, K. (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *Embo J*, **19**, 1777-1783.
- Schapira, A.H. (2006) Mitochondrial disease. *Lancet*, **368**, 70-82.
- Schwartz, M. and Vissing, J. (2002) Paternal inheritance of mitochondrial DNA. *N Engl J Med*, **347**, 576-580.
- Seki, M., Marini, F. and Wood, R.D. (2003) POLQ (Pol theta), a DNA polymerase and DNA-dependent ATPase in human cells. *Nucleic Acids Res*, **31**, 6117-6126.
- Shadel, G.S. and Clayton, D.A. (1997) Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem*, **66**, 409-435.
- Shapiro, T.A. and Englund, P.T. (1995) The structure and replication of kinetoplast DNA. *Annu Rev Microbiol*, **49**, 117-143.
- Sharief, F.S., Vojta, P.J., Ropp, P.A. and Copeland, W.C. (1999) Cloning and chromosomal mapping of the human DNA polymerase theta (POLQ), the eighth human DNA polymerase. *Genomics*, **59**, 90-96.
- Shcherbakova, P.V., Bebenek, K. and Kunkel, T.A. (2003) Functions of eukaryotic DNA polymerases. *Sci Aging Knowledge Environ*, **2003**, RE3.
- Shutt, T.E. and Gray, M.W. (2006) Twinkle, the mitochondrial replicative DNA helicase, is widespread in the eukaryotic radiation and may also be the mitochondrial DNA primase in most eukaryotes. *J Mol Evol*, **62**, 588-599.
- Singleton, M.R., Sawaya, M.R., Ellenberger, T. and Wigley, D.B. (2000) Crystal structure of T7 gene 4 ring helicase indicates a mechanism for sequential hydrolysis of nucleotides. *Cell*, **101**, 589-600.
- Spelbrink, J.N., Li, F.Y., Tiranti, V., Nikali, K., Yuan, Q.P., Tariq, M., Wanrooij, S., Garrido, N., Comi, G., Morandi, L., Santoro, L., Toscano, A., Fabrizi, G.M., Somer, H., Croxen, R., Beeson, D., Poulton, J., Suomalainen, A., Jacobs, H.T., Zeviani, M. and Larsson, C. (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat Genet*, **28**, 223-231.
- Spinazzola, A. and Zeviani, M. (2005) Disorders of nuclear-mitochondrial intergenomic signaling. *Gene*, **354**, 162-168.

- Stano, N.M., Jeong, Y.J., Donmez, I., Tummalapalli, P., Levin, M.K. and Patel, S.S. (2005) DNA synthesis provides the driving force to accelerate DNA unwinding by a helicase. *Nature*, **435**, 370-373.
- Sutovsky, P., Moreno, R.D., Ramalho-Santos, J., Dominko, T., Simerly, C. and Schatten, G. (1999) Ubiquitin tag for sperm mitochondria. *Nature*, **402**, 371-372.
- Taanman, J.W. (1999) The mitochondrial genome: structure, transcription, translation and replication. *Biochim Biophys Acta*, **1410**, 103-123.
- Tabor, S., Huber, H.E. and Richardson, C.C. (1987) Escherichia coli thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. *J Biol Chem*, **262**, 16212-16223.
- Tabor, S. and Richardson, C.C. (1981) Template recognition sequence for RNA primer synthesis by gene 4 protein of bacteriophage T7. *Proc Natl Acad Sci U S A*, **78**, 205-209.
- Takamatsu, C., Umeda, S., Ohsato, T., Ohno, T., Abe, Y., Fukuoh, A., Shinagawa, H., Hamasaki, N. and Kang, D. (2002) Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep*, **3**, 451-456.
- Tanguy Le Gac, N., Villani, G., Hoffmann, J.S. and Boehmer, P.E. (1996) The UL8 subunit of the herpes simplex virus type-1 DNA helicase-primase optimizes utilization of DNA templates covered by the homologous single-strand DNA-binding protein ICP8. *J Biol Chem*, **271**, 21645-21651.
- Tapper, D.P. and Clayton, D.A. (1981) Mechanism of replication of human mitochondrial DNA. Localization of the 5' ends of nascent daughter strands. *J Biol Chem*, **256**, 5109-5115.
- Tapper, D.P. and Clayton, D.A. (1982) Precise nucleotide location of the 5' ends of RNA-primed nascent light strands of mouse mitochondrial DNA. *J Mol Biol*, **162**, 1-16.
- Tiranti, V., Savoia, A., Forti, F., D'Apolito, M.F., Centra, M., Rocchi, M. and Zeviani, M. (1997) Identification of the gene encoding the human mitochondrial RNA polymerase (h-mtRPOL) by cyberscreening of the Expressed Sequence Tags database. *Hum Mol Genet*, **6**, 615-625.
- Topper, J.N. and Clayton, D.A. (1989) Identification of transcriptional regulatory elements in human mitochondrial DNA by linker substitution analysis. *Mol Cell Biol*, **9**, 1200-1211.
- Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly, Y.M., Gidlof, S., Oldfors, A., Wibom, R., Tornell, J., Jacobs, H.T. and Larsson, N.G. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature*, **429**, 417-423.
- Tyynismaa, H., Sembongi, H., Bokori-Brown, M., Granycome, C., Ashley, N., Poulton, J., Jalanko, A., Spelbrink, J.N., Holt, I.J. and Suomalainen, A. (2004) Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number. *Hum Mol Genet*, **13**, 3219-3227.
- Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M., Elsas, L.J., 2nd and Nikoskelainen, E.K. (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science*, **242**, 1427-1430.
- Van Dyck, E., Foury, F., Stillman, B. and Brill, S.J. (1992) A single-stranded DNA binding protein required for mitochondrial DNA replication in *S. cerevisiae* is homologous to *E. coli* SSB. *Embo J*, **11**, 3421-3430.
- Van Goethem, G., Martin, J.J. and Van Broeckhoven, C. (2002) Progressive external ophthalmoplegia and multiple mitochondrial DNA deletions. *Acta Neurol Belg*, **102**, 39-42.



- Van Goethem, G., Martin, J.J. and Van Broeckhoven, C. (2003) Progressive external ophthalmoplegia characterized by multiple deletions of mitochondrial DNA: unraveling the pathogenesis of human mitochondrial DNA instability and the initiation of a genetic classification. *Neuromolecular Med*, **3**, 129-146.
- Wang, Y. and Bogenhagen, D.F. (2006) Human mitochondrial DNA nucleoids are linked to protein folding machinery and metabolic enzymes at the mitochondrial inner membrane. *J Biol Chem*, **281**, 25791-25802.
- Wang, Y., Lyu, Y.L. and Wang, J.C. (2002) Dual localization of human DNA topoisomerase III $\alpha$  to mitochondria and nucleus. *Proc Natl Acad Sci U S A*, **99**, 12114-12119.
- Vishwanatha, J.K. and Baril, E.F. (1986) Resolution and purification of free primase activity from the DNA primase-polymerase  $\alpha$  complex of HeLa cells. *Nucleic Acids Res*, **14**, 8467-8487.
- Wolstenholme, D.R. (1992) Animal mitochondrial DNA: structure and evolution. *Int Rev Cytol*, **141**, 173-216.
- Wong, T.W. and Clayton, D.A. (1985) Isolation and characterization of a DNA primase from human mitochondria. *J Biol Chem*, **260**, 11530-11535.
- Wu, C.A., Zechner, E.L., Hughes, A.J., Jr., Franden, M.A., McHenry, C.S. and Marians, K.J. (1992) Coordinated leading- and lagging-strand synthesis at the Escherichia coli DNA replication fork. IV. Reconstitution of an asymmetric, dimeric DNA polymerase III holoenzyme. *J Biol Chem*, **267**, 4064-4073.
- Xu, B. and Clayton, D.A. (1995) A persistent RNA-DNA hybrid is formed during transcription at a phylogenetically conserved mitochondrial DNA sequence. *Mol Cell Biol*, **15**, 580-589.
- Yakubovskaya, E., Chen, Z., Carrodeguas, J.A., Kisker, C. and Bogenhagen, D.F. (2006) Functional human mitochondrial DNA polymerase  $\gamma$  forms a heterotrimer. *J Biol Chem*, **281**, 374-382.
- Yang, C., Curth, U., Urbanke, C. and Kang, C. (1997) Crystal structure of human mitochondrial single-stranded DNA binding protein at 2.4 Å resolution. *Nat Struct Biol*, **4**, 153-157.
- Yang, M.Y., Bowmaker, M., Reyes, A., Vergani, L., Angeli, P., Gringeri, E., Jacobs, H.T. and Holt, I.J. (2002) Biased incorporation of ribonucleotides on the mitochondrial L-strand accounts for apparent strand-asymmetric DNA replication. *Cell*, **111**, 495-505.
- Yasukawa, T., Reyes, A., Cluett, T.J., Yang, M.Y., Bowmaker, M., Jacobs, H.T. and Holt, I.J. (2006) Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *Embo J*, **25**, 5358-5371.
- Yasukawa, T., Yang, M.Y., Jacobs, H.T. and Holt, I.J. (2005) A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. *Mol Cell*, **18**, 651-662.
- Yuzaki, M., Ohkoshi, N., Kanazawa, I., Kagawa, Y. and Ohta, S. (1989) Multiple deletions in mitochondrial DNA at direct repeats of non-D-loop regions in cases of familial mitochondrial myopathy. *Biochem Biophys Res Commun*, **164**, 1352-1357.
- Zeviani, M. and Di Donato, S. (2004) Mitochondrial disorders. *Brain*, **127**, 2153-2172.
- Zeviani, M., Servidei, S., Gellera, C., Bertini, E., DiMauro, S. and DiDonato, S. (1989) An autosomal dominant disorder with multiple deletions of mitochondrial DNA starting at the D-loop region. *Nature*, **339**, 309-311.
- Zhang, H., Barcelo, J.M., Lee, B., Kohlhagen, G., Zimonjic, D.B., Popescu, N.C. and Pommier, Y. (2001) Human mitochondrial topoisomerase I. *Proc Natl Acad Sci U S A*, **98**, 10608-10613.

